

STUDIES OF THE BIOSYNTHESIS OF

HISTIDINE IN NEUROSPORA CRASSA

by

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This thesis describes the results of my own work, performed under the supervision of Dr E.H. Creaser.

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The photographic prints for this thesis were prepared by Mr D. Parr, whose help is greatly appreciated, and I wish to thank Mrs F. Swan for typing the thesis.

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SYNOPSIS

This thesis describes an investigation into a special characteristic of histidine biosynthesis in Neurospora crassa; namely that a short genetic segment, the his-3 locus, controls three reactions, steps 2, 3 and 10, in the histidine pathway of this organism. These reactions are catalysed by the enzymes PRATP pyrophosphohydrolase, PRAMP 1-6 cyclohydrolase and histidinol dehydrogenase.

The known genetic and biochemical properties of his-3 mutants are discussed, and it is shown that the present genetic and biochemical evidence is open to more than one interpretation. Methods are proposed for distinguishing between these alternatives.

A method is proposed for the assay of PRATP pyrophosphohydrolase and PRAMP cyclohydrolase.

Experiments are described to determine whether histidinol dehydrogenase is a tri-functional protein (a protein carrying histidinol dehydrogenase activity had been obtained in a highly purified form before this investigation was begun). It is shown that PRATP pyrophosphohydrolase, PRAMP cyclohydrolase and histidinol

dehydrogenase fractionate together during the early stages of the purification of histidinol dehydrogenase, but that the purified histidinol dehydrogenase does not carry the former two activities. A technique is described which restores some pyrophosphohydrolase and cyclohydrolase activity to purified histidinol dehydrogenase.

A purification procedure is described which results in a protein which carries all three enzyme activities in approximately the same ratio as is found in the crude protein extract. Purity criteria are given and are discussed.

Some chemical and physical properties of the tri-functional protein are described, and a comparison is made of the tri-functional protein and its uni-functional counterpart, histidinol dehydrogenase. Attempts are made to infer the sub-unit structure of the tri-functional protein.

Catalysis of reactions 2, 3 and 10 in the histidine pathway of Neurospora crassa is compared with the catalysis of the same reactions in other organisms. This multi-functional protein is compared with other multi-functional proteins and the role of multi-functional proteins is discussed. Suggestions for further work are made.

PREFACE

Figures and tables are identified by two numbers. The second number indicates the chapter, and the first indicates the number of the figure or table within the chapter. Thus, Figure 1.4 and Table 1.4 are the first figure and the first table respectively in Chapter 4.

Abbreviations used are:

ATP, ADP, AMP	Adenosine mono, di and triphosphate
NAD	Nicotinamide-adenine dinucleotide
DEAE, TEAE	Di and triethylaminoethyl
CM	Carboxymethyl
tris	2-amino-2-hydroxymethylpropane-1,3-diol
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
sRNA	Soluble (transfer) RNA
DNase	Deoxyribonuclease
RNase	Ribonuclease

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CHAPTER 1

Introduction

The relationship between the genotype and phenotype

The introduction of auxotrophic mutants of micro-organisms as experimental material by Beadle and Tatum (1945), and the subsequent combination of biochemical and genetic techniques in the study of gene action, has led to our present understanding of the mechanism by which the genetic material directs the production of enzymes. The following facts are now well established.

- (1) That the genetic material is nucleic acid, and with the exception of some viruses, the primary genetic material is DNA.
- (2) That where DNA is the primary genetic material the genetic code is transcribed into a molecule of messenger RNA by specific nucleotide base pairing.
- (3) That the linear sequence of nucleotides in messenger RNA is translated into a linear sequence of amino acids by specific base pairing of aminoacyl-s-RNA molecules.
- (4) That the genetic code is triplet, each amino acid being specified by a sequence of three nucleotides of four sorts.

The way in which the DNA is organised in the cell has proved a rather intractable problem, but there is evidence that at least in some bacteriophages the total DNA content exists as a continuous sequence of nucleotides (Thomas, Ritchie and MacHattie, 1967). However, the genetic information is translated into a discontinuous sequence of amino acids, and the DNA can therefore be divided into segments so that each segment contains the code for one amino acid sequence or polypeptide. Such a segment of DNA may be termed a 'structural gene'. The general relationship between genotype and phenotype is shown in fig. 1.1. The diagram has been drawn to show a unit relationship between structural gene, messenger RNA, amino acid sequence, enzyme protein, and enzyme catalysed reaction.

Exceptions to the general relationship

If the relationship shown in fig. 1.1 were always true, then a single mutation could only affect one species of polypeptide, and hence only one enzyme catalysed reaction. Furthermore, a particular enzyme catalysed reaction could be affected by mutations in only one structural gene. Thus, as represented in fig. 1.1, the structural gene is the genetic unit of expression or function. That this is not always the case is

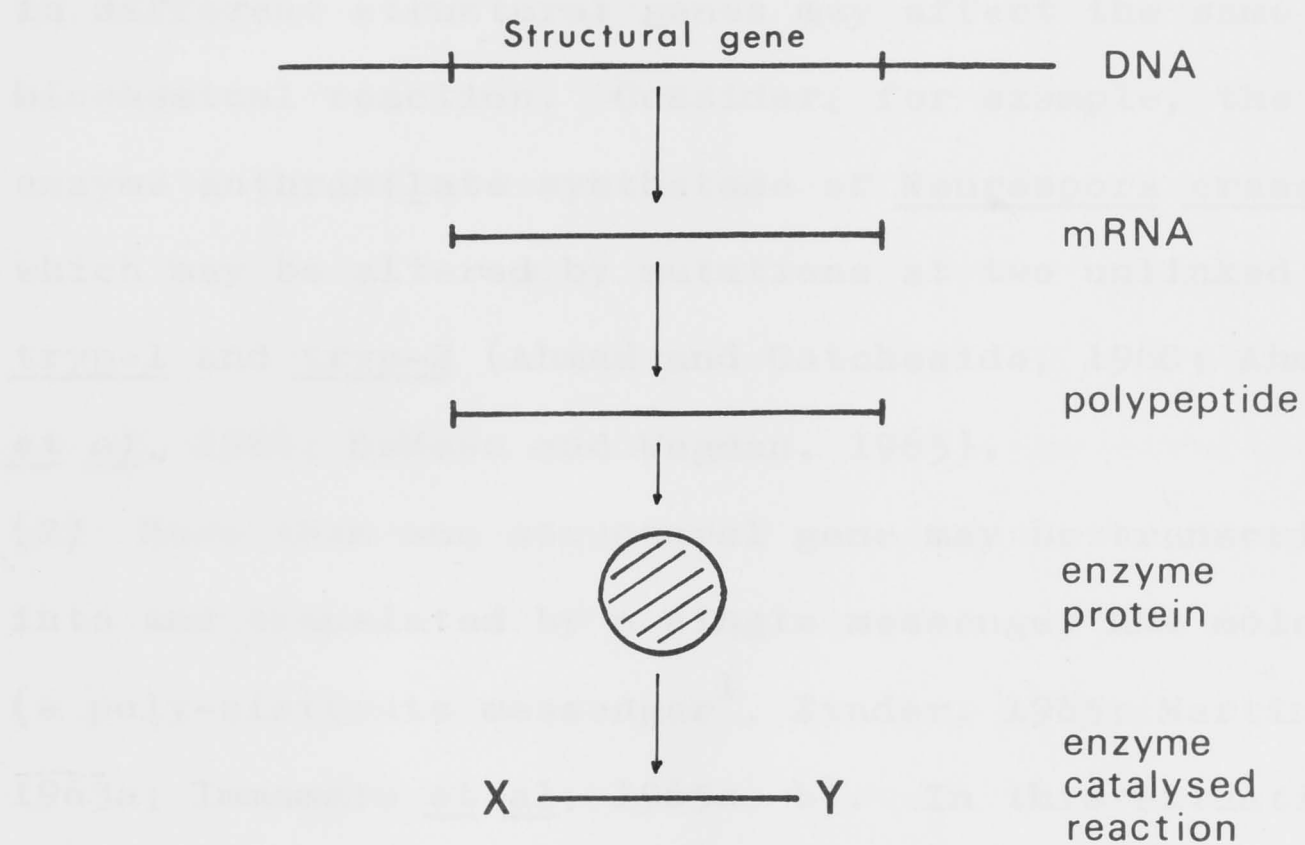


Figure 1.1 The general relationship between the genotype and the phenotype.

apparent from the following exceptions to the general relationship shown in fig. 1.1.

(1) A particular reaction may be catalysed by a heteropolymeric enzyme; an enzyme composed of more than one type of polypeptide. In this case mutations in different structural genes may affect the same biochemical reaction. Consider, for example, the enzyme anthranilate synthetase of Neurospora crassa, which may be altered by mutations at two unlinked loci, tryp-1 and tryp-2 (Ahmad and Catchside, 1960; Ahmad et al, 1964; DeMoss and Wegman, 1965).

(2) More than one structural gene may be transcribed into and translated by a single messenger RNA molecule (a poly-cistronic messenger¹, Zinder, 1965; Martin, 1963a; Immamoto et al, 1965a, b). In this situation a mutation may affect the translation of all or part of the messenger, so that a single mutation may affect

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The term 'cistron' was originally introduced by Benzer (1958), and was defined as that segment of the genome in which mutants failed to complement one another. At that time the term was thought to have a mechanistic meaning because complementation was thought to occur only if the two mutants had alterations in different polypeptides. The cistron was therefore the genetic unit which specified a polypeptide. The discovery of interallelic complementation rendered the original definition of the term meaningless, but the term has outlived its definition, and now has the same meaning as structural gene.

several polypeptides and hence several enzyme catalysed reactions. Mutations which impair the translation of messenger molecules arise by base pair insertions or deletions ('frame shift mutations'), or by base pair substitutions giving rise to chain terminating triplets (Crick et al., 1961; Sarabhai et al., 1964; Martin et al., 1966; Whitfield, Martin & Ames, 1966; Yanofsky & Ito, 1966), and mutants of this sort are selected with high frequency in the histidine operon of Salmonella typhimurium (Ames and Hartman, 1963). A characteristic of such mutations is that they affect the structural gene in which they occur, and any structural genes translated subsequently. That section of the messenger which is translated prior to the site of mutation is translated normally. Thus the genetic material on only one side of the mutation is affected, and since translation is always in the same direction (Smith et al., 1966), this is the same side for all such mutations in a particular unit of translation. Thus these mutations exhibit a polarised effect, and are often referred to as 'polarity mutations'.

(3) A single mutation may also affect more than one enzyme catalysed reaction if one species of polypeptide is involved in the catalysis of more than one biochemical

reaction. An example is provided by the his-B gene of Salmonella typhimurium, which appears to specify a polypeptide with two enzymic activities, imidazoleglycerol phosphate dehydrase and histidinol phosphate phosphatase (Loper, 1961; Loper et al, 1964).

It is clear that, despite our present understanding of the mechanism of gene expression, the existence of these exceptions to the general relationship shown in fig. 1.1 introduces a measure of uncertainty into the interpretation of genetic information in terms of proteins and enzyme reactions, because the relationship between mutations and enzymic defects is not always a simple one.

Gene-enzyme relationships in the biosynthesis of histidine in Neurospora crassa

(2) Histidine mutants of *Neurospora* may be divided into seven distinct groups on the basis of linkage and complementation studies (Haas, Mitchell, Ames and Mitchell, 1952; Mathieson and Catcheside, 1955; Webber and Case, 1960; Catcheside, 1960). Mutants in each group are closely linked to all other mutants in the same group, and some mutants in each group will not complement with any mutants in the same group, but will complement with all mutants in other groups. The seven

groups are designated his-1 to his-7. With the exception of his-3 mutants, all mutants in a particular group were found to have the same biochemical characteristics, each group being characterised by the loss of ability to catalyse one of the reactions in the histidine biosynthetic pathway (Haas et al., 1952; Mathieson and Catcheside, 1955; Ames and Horecker, 1956; Ames, 1957a,b; Catcheside, 1960; Webber and Case, 1960; Webber, 1960; Ahmed, Case and Giles, 1964). His-3 mutants, however, were found to have diverse biochemical characteristics, and could be divided into three groups on this basis (Haas et al., 1952; Webber and Case, 1960; Webber, 1960; Catcheside, 1960, 1965):

- (1) those which lacked the enzyme histidinol dehydrogenase and accumulated histidinol;
- (2) those which contained histidinol dehydrogenase and accumulated no imidazole precursors of histidine;
- (3) those which lacked histidinol dehydrogenase, but did not accumulate histidinol.

From this result it was apparent that the his-3 locus controlled at least two reactions in the histidine biosynthetic pathway (see fig. 2.1); (a) the oxidation of histidinol to histidine and (b) some reaction or reactions earlier in the pathway than D-erythro-

Abbreviations used for intermediates in the histidine pathway

PRPP	5'phosphoribosyl-1-pyrophosphate
PRATP	N-1-(5'phosphoribosyl)-ATP
PRAMP	N-1-(5'phosphoribosyl)-AMP
AIC-R-P	5-amino-1-(5'phosphoribosyl)-4-imidazolecarboxamide
BBM I	N-(5'phospho-D-ribosylformimino)-5-amino-1-ribosyl-(5"triphosphate)-4-imidazolecarboxamide
BBM II	N-(5'phospho-D-ribosylformimino)-5-amino-1-(5"phosphoribosyl)-4-imidazolecarboxamide
BBM III	N-(5'phospho-D-ribulosylformimino)-5-amino-1-(5"phosphoribosyl)-4-imidazolecarboxamide

Note that BBM I is not a histidine pathway intermediate. The compound results from the hydrolysis of the 1,6 bond of the adenine ring prior to the removal of pyrophosphate. This is apparently due to the non-specific action of PRAMP cyclohydrolase on PRATP. As a result, mutants which lack pyrophosphohydrolase but contain cyclohydrolase accumulate BBM I.

Systemic enzyme names

IGP dehydrase	Imidazoleglycerolphosphate hydro-lyase (E.C.4.2.1.19).
IAP aminotransferase	L-histidinolphosphate:2-oxoglutarate aminotransferase (E.C.2.6.1.9).
HP phosphatase	L-histidinolphosphate phosphohydrolase (E.C.3.1.3.15).
Histidinol dehydrogenase	L-histidinol:NAD oxidoreductase (E.C.1.1.1.23).

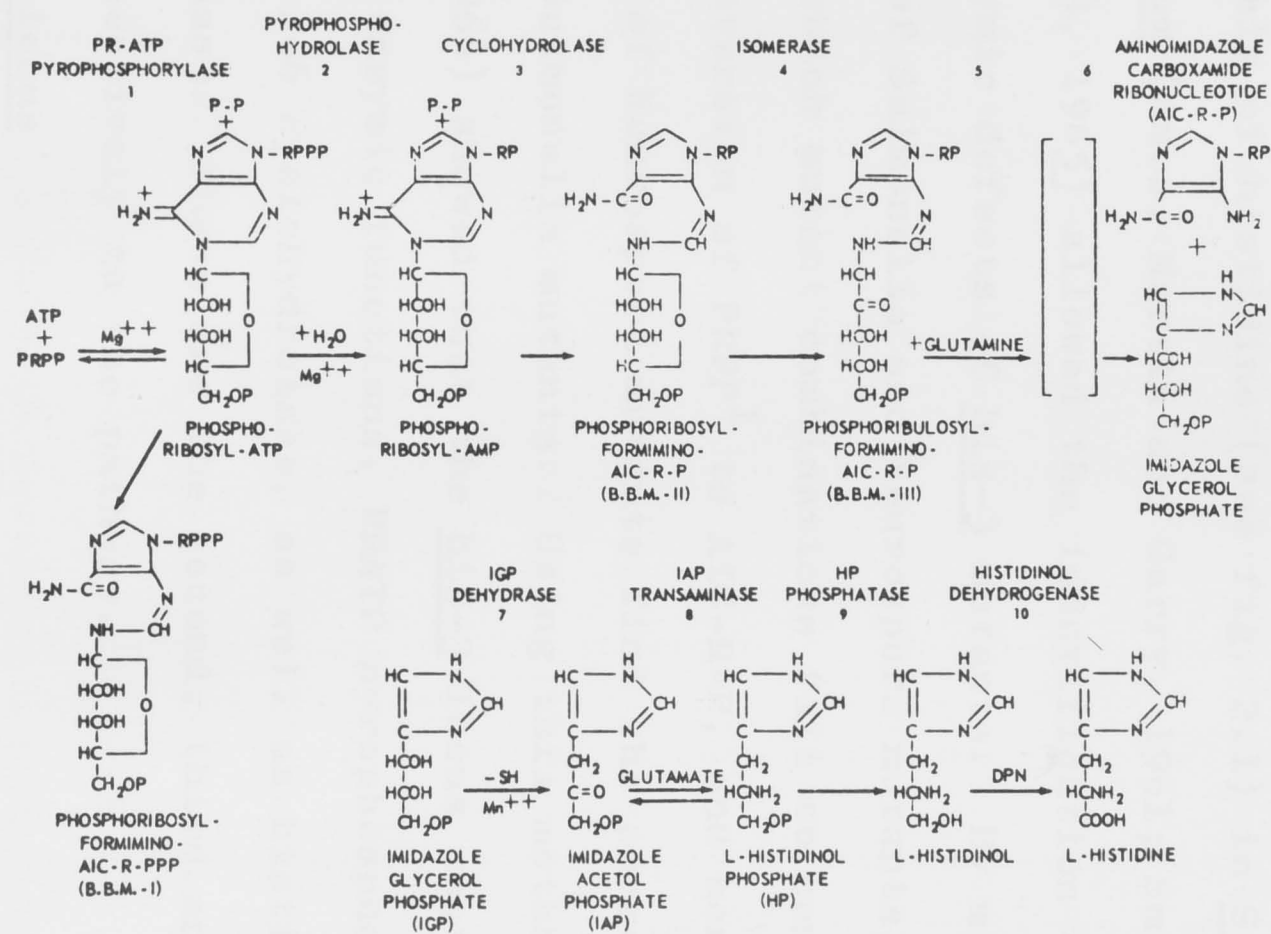


Figure 2.1 The histidine biosynthetic pathway

imidazoleglycerol phosphate. Thus group (1) mutants lacked histidinol dehydrogenase, group (2) mutants lacked an early enzyme and group (3) mutants lacked both enzymes.

The elucidation of the early steps in the biosynthesis of histidine (see fig. 2.1) in Salmonella typhimurium (Ames, Martin and Garry, 1961; Smith and Ames, 1964, 1965) allowed the identification of the early enzymic defects of his-3 mutants. By mixing extracts of Salmonella and Neurospora mutants, it can be found which mutant combinations fail to complement in the conversion of PRPP¹ to AIC-R-P, and hence for any group of Neurospora mutants find the corresponding group of Salmonella mutants. Using this method Ahmed et al. (1964) showed that the his-3 locus controlled two early enzymic functions, PRATP pyrophosphohydrolase and PRAMP 1-6 cyclohydrolase, as well as histidinol dehydrogenase. These are the second, third and tenth steps respectively in the pathway.

The his-3 locus

Genetic analysis of the his-3 locus has been carried out by a number of workers; Catcheside (1960,

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Abbreviations used for histidine pathway intermediates are given facing Fig. 2.1.

1965); Ahmed et al. (1964); Giles (1965), and Webber (1959, 1960, 1965a,b). There is good general agreement between the results of these workers. Genetic, complementation and biochemical data according to Catcheside (1965) and according to Ahmed et al. (1964) are shown in figs. 3.1 and 4.1. The general features of both sets of data are as follows.

(1) It appears that the his-3 locus can be differentiated on the basis of function. Mutations which result only in the loss of histidinol dehydrogenase activity are located on the right hand (distal) side of both genetic maps. Catcheside's data does not distinguish between mutants which lack PRATP pyrophosphohydrolase and mutants which lack PRAMP cyclohydrolase. However, mutations which result in the loss of one or both of these activities, but not in the loss of histidinol dehydrogenase, are located on the left hand (proximal) side of the map. Ahmed et al. (1964) obtained a similar result, but they were able to distinguish between mutants which lacked PRATP pyrophosphohydrolase and those which lacked PRAMP cyclohydrolase, and found that these two groups of mutants were located at discrete parts of the genetic map. Thus Ahmed et al. (1964) recognised three distinct segments within the his-3

(i)	(ii)	(iii)	(iv)	(v)
A	—————	0	0	52
B	———	0	+	1
C	—————	0	0	13
D	—————	0	+	1
E	———	0	+	1
F	———	0	+	9
G	—————	+	0	21
H	———	+	0	8
I	—————	+	0	8
J	———	+	0	1
K	———	+	0	9

Properties of his-3 mutants of Neurospora crassa: (i) designation of groups of mutants; (ii) complementation map; (iii) ability to accumulate histidinol; (iv) occurrence of histidinol dehydrogenase; (v) number of mutants in each group.



Genetic map of his-3 showing the mutation sites identified by mutant group letters. The extreme A mutants are respectively K474 and K504. The map is not strictly to scale, the breaks to left and right representing long intervals.

Figure 3.1 Genetic and biochemical properties of his-3 mutants according to Catcheside (1965).

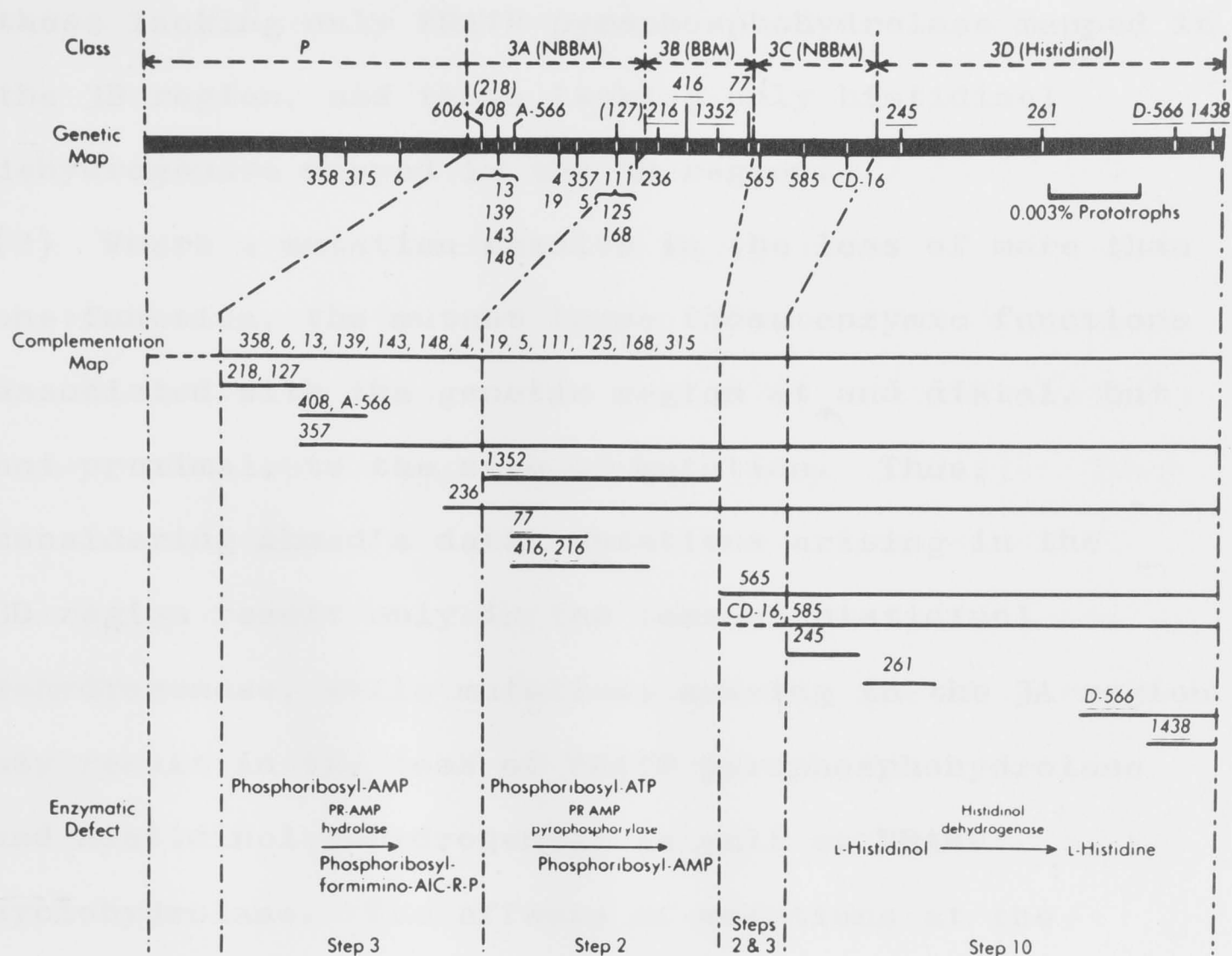


Figure 3. Correlation of the genetic map of the *histidine-3* region with the complementation map and with enzymatic defects. The numerals above the genetic map refer to complementing mutants, while those below designate completely or partially noncomplementing mutants (referred to as pleiotropic or polarity mutants). The region designated *P* may correspond to the "promoter"¹² on the genetic map, which cannot be distinguished as a separate region on the complementation map because of its pleiotropic effects. Mutant numbers that are underlined indicate accumulation of either BBM or histidinol; numbers in parentheses (or brackets) refer to mutants whose locations with respect to closely adjacent mutants on the genetic map are not yet precisely established. Data in part from Webber.^{7,9}

Figure 4.1 Genetic and biochemical properties of *his-3* mutants according to Ahmed *et al* (1964).

locus, his-3A, his-3B and his-3D. Mutants lacking only PRAMP cyclohydrolase mapped in the his-3A region; those lacking only PRATP pyrophosphohydrolase mapped in the 3B region, and those lacking only histidinol dehydrogenase mapped in the 3D region.

(2) Where a mutation results in the loss of more than one function, the mutant loses those enzymic functions associated with the genetic region at and distal, but not proximal, to the site of mutation. Thus, considering Ahmed's data, mutations arising in the 3D region result only in the loss of histidinol dehydrogenase, while mutations arising in the 3A region may result in the loss of PRATP pyrophosphohydrolase and histidinol dehydrogenase as well as PRAMP cyclohydrolase. The effects of mutations at the his-3 locus are therefore polarised in the distal direction.

(3) In general the complementation behaviour of mutants is in agreement with the behaviour which might be predicted from a knowledge of their enzymic defects. Mutants which lack one of the three enzymic functions complement strongly with mutants which lack one or both of the remaining two functions. Complementation also occurs between mutants with the same enzymic defect, but

in these cases complementation is usually weak. Both sets of data include a large group of non-complementing mutants, and these are situated on the proximal side of the genetic map.¹

On the basis of these generalisations it is tempting to conclude that the his-3 locus is analogous in some respects to a bacterial operon (Jacob and Monod, 1961; Tomkins and Ames, 1967), and this was the interpretation favoured by Ahmed et al. (1964). They considered that the data could be best explained if the his-3 locus consisted of three structural genes represented by the genetic regions his-3A, 3B and 3D. His-3A specifies PRAMP cyclohydrolase, 3B specifies PRATP pyrophosphohydrolase and 3D specifies histidinol dehydrogenase, but the three structural genes are transcribed into and translated by a single messenger RNA molecule. Two classes of mutations may then account for the characteristics of his-3 mutants.

(1) Mutations which result in an amino acid substitution in the polypeptide specified by the structural gene in which they arise (mis-sense mutations). They have no quantitative or qualitative effect on the

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A general discussion of the interpretation of complementation data is given in Appendix I.

translation of neighbouring structural genes, and therefore affect only one polypeptide.

(2) 'Frame shift' or 'chain terminating' mutations.

Such mutations alter the translation of the structural gene in which they arise, and of any structural gene translated subsequently. They may therefore affect the production of more than one species of polypeptide.

It is clear that this hypothesis accounts adequately for the characteristics of most his-3 mutants. A mis-sense mutation in the 3A region would result in the loss of only PRAMP cyclohydrolase activity, while a frame shift mutation or chain terminating mutation in this region would result in the loss of all three activities. Both kinds of mutation arising in the 3D region would result only in the loss of histidinol dehydrogenase activity. The analogy with the bacterial operon is supported by the recognition, by Ahmed et al. (1964), of a segment, proximal to the 3A region, in which all the observed mutations result in the loss of all three activities. This segment might be considered analogous to the promoter region of a bacterial operon (Jacob, Ullman and Monod, 1964).

Ahmed et al. (1964) attempted to extend the evidence that the his-3 locus contained more than one structural

gene by studying the nature of complementation between his-3 mutants. They found that heterocaryons formed from two 3D mutants grew very poorly in the absence of histidine, and concluded that complementation between his-3D mutants was interallelic. 3A/3D and 3B/3D heterocaryons, however, grew as well as the wild type organism, suggesting that these heterocaryons contained a 'wild type' enzyme complement, and hence that complementation between these mutants was intra-allelic. It has already been stated, however, that 3A mutants contain histidinol dehydrogenase, and that 3D mutants contain PRAMP cyclohydrolase. Thus, although the observation that 3A/3D heterocaryons are functionally wild type is evidence that the his-3 locus is polycistronic, it is not additional evidence, but merely the corollary of the observation that the locus may be differentiated on the basis of the enzymic defects caused by mutation.

The same problem was approached by investigating the properties of histidinol dehydrogenase from heterocaryons formed from various combinations of his-3 mutants (Ahmed et al, 1964). 3D/3D heterocaryons were found to contain a histidinol dehydrogenase which was much more heat sensitive than the enzyme in the wild

type organism, a result which was also reported by McGlothlen (1965). 3A/3D and 3B/3D heterocaryons, however, contained a histidinol dehydrogenase which was very similar to the wild type enzyme in its heat inactivation properties, suggesting that these heterocaryons contain 'wild type' histidinol dehydrogenase, and hence are formed through intra-allelic complementation. These experiments were not reported in detail, however, and the properties of the histidinol dehydrogenase from the mutants used to make the heterocaryons were not given. In fact this information is essential for the full interpretation of Ahmed's result. If a 3A mutant contains histidinol dehydrogenase which is significantly different from that of the wild type organism, then the his-3 locus cannot produce three independent enzymes, because a mutation in the 3A region should not alter the properties of an independent enzyme supposedly specified by the 3D region. Indeed, if the 3A mutant used by Ahmed et al. (1964) in the experiments described above, did contain histidinol dehydrogenase which had significantly different heat inactivation properties from the wild type enzyme, then Ahmed's result must be interpreted as meaning that the his-3 locus specifies

more than one type of polypeptide, but that these polypeptides associate to form a multi-functional heteropolymeric protein.

The evidence that the his-3 locus consists of several structural genes translated by a single messenger molecule, may be reduced to the following statements.

- (1) That the locus controls three enzymic functions.
- (2) That, in general, mutations resulting in the loss of a particular single enzymic function are located together on the genetic map, but are separated from mutations resulting in the loss of other single functions.
- (3) That where a mutation results in multiple enzymic defects, those enzymic functions are lost which are associated with the genetic region distal to the site of mutation.

In fact the data both of Catcheside (1965) and of Ahmed et al. (1964) show exceptions to the general statements made in (2) and (3) above. Catcheside's genetic map shows one G group mutant (which lacks only histidinol dehydrogenase) which is located proximally to C and F group mutants (which lack one or both of the other two functions but contain histidinol dehydrogenase).

Thus that section of the map responsible for histidinol dehydrogenase activity overlaps that section responsible for the other two functions, and on this evidence the his-3 locus cannot be divided into discrete sections. Ahmed's map shows a group of mutants, the 3C mutants, which are located at the centre of the map, but which have lost all three enzymic functions. Mutations in the 3C region therefore lose enzymic functions associated with the genetic regions both distal and proximal to the site of mutation. The existence of this group can be explained in the context of the poly-cistronic messenger hypothesis by supposing that the 3C region specifies a polypeptide which is essential both for PRATP pyrophosphohydrolase activity and for PRAMP cyclohydrolase activity. The loss of histidinol dehydrogenase activity can be explained by assuming that all the 3C mutants so far obtained are of the polarity type, and that the 3D region is therefore mis-translated or not translated in these mutants (Ahmed et al., 1964). This explanation of the nature of these mutants is consistent with the fact that they will complement with 3A and 3B mutants, but not with 3D mutants. Recent work has established that another non-complementing mutant, K475, is located in the distal part of the map, so that the argument used above is still valid (K.K. Jha, personal communication).

The situation is further complicated by the position of K504 (Catcheside's data). This mutant is non-complementing, and therefore lacks all three enzymic functions, but is located at the extreme right of the genetic map.¹ If the his-3 locus consists of more than one structural gene, then the behaviour of this mutant can only be explained if, in this single case, translation occurs from right to left. Furthermore, it should be noted that non-complementing mutants comprise about 45% of the his-3 mutants obtained (Catcheside's data). Yet the poly-cistronic messenger hypothesis predicts that non-complementing mutants can only arise by frame shift or chain terminating mutations in the first structural gene (translated) in the locus, and it seems unlikely that 45% of the his-3 mutants obtained would be of this type. In general, Catcheside's results do not fit as well to the poly-cistronic messenger hypothesis as do those of Ahmed et al. (1964), and this led Catcheside (1965) to

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In fact the position of mutant K504 is now in doubt, and recent evidence suggests that this mutant is located in the proximal part of the genetic map (D.G. Catcheside and K.K. Jha, personal communication). However, recent work has established that another non-complementing mutant, K474, is located in the distal part of the map, so that the argument used above is still valid (K.K. Jha, personal communication).

propose an alternative hypothesis. He proposed that the his-3 locus consisted of a single structural gene which specified a single polypeptide with the potential to catalyse the pyrophosphohydrolase, cyclohydrolase and dehydrogenase reactions.

The difference between these two hypotheses is that one seeks to explain the data in terms of the gene and its translation, whereas the other seeks to explain the data in terms of the possible structure and function of the protein product. A diagrammatic representation of the two hypotheses is shown in fig. 5.1. Catcheside's interpretation is, perhaps, unattractive because it demands that a single polypeptide species be capable of catalysing three chemically unrelated reactions. However, the general principle that one type of polypeptide catalyses one reaction is based largely on negative evidence, and we cannot rule out the possibility that genetic data of the sort obtained from the his-3 locus might be due to the complexity of a single product.

It has been demonstrated that chemical manipulation of parts of a protein need not result in loss of activity (Kalnitsky and Anderson, 1955; Hill and Smith, 1955), so that it appears that some parts of the polypeptide are more important than others in maintaining the

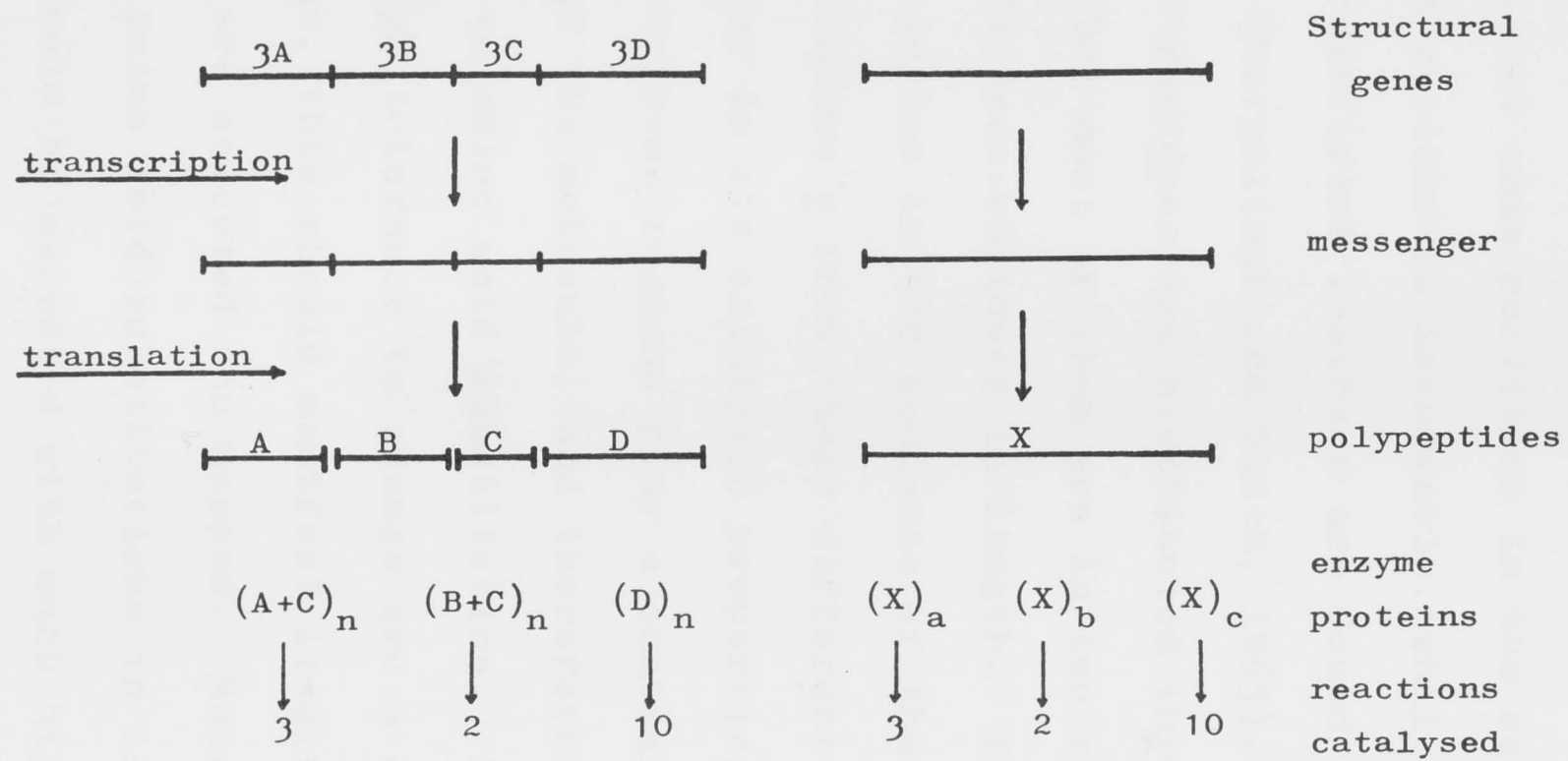


Figure 5.1 Mechanisms of expression of the *his-3* locus.

Left: proposed by Ahmed et al (1964).

Right: proposed by Catchside (1965).

functional integrity of the molecule. The same conclusion may be drawn from a comparison of the amino acid sequence of cytochrome c from a wide variety of organisms. At some positions in the sequence the amino acid residue is invariable, while at other positions different residues are found in different organisms (Margoliash and Smith, 1965). The invariable residues are distributed throughout the sequence, but most of them are in two regions, each of about fifteen residues in length. There is very little variation in the sequence of these regions, and, since cytochrome c from these different organisms is very similar in its catalytic properties, it can be argued that these regions play a central role in the function of the molecule, and therefore have a low tolerance to amino acid substitution. If regions of low and high tolerance to change are a characteristic of proteins, this should manifest itself when mutants of a gene are selected and mapped. Mutations which result in amino acid substitutions in low tolerance regions should be selected with much higher frequency than those which cause substitutions in other parts of the molecule, and the genetic map should show a high

density of mutations in certain regions.¹ It seems likely therefore, that, given a protein with several catalytic functions, different parts of the molecule might be important for the maintenance of different functions, and this be reflected in the fact that mutations resulting in the loss of one of the functions would tend to be located in a different section of the map from mutations resulting in the loss of another function. Mutations which resulted in more general damage, such as chain termination mutations, frame shift mutations and mutations resulting in amino acid substitutions at positions important in maintaining the tertiary and quaternary structure of the molecule, would result in the loss of more than a single function, and would be more widely distributed through the gene (Langridge, 1968). Provided that the concept of a protein with multiple catalytic functions is acceptable, the his-3 genetic data can be adequately explained by such reasoning.

It is clear that detailed genetic analysis, combined with preliminary biochemical data, has failed to

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That this is often not the case is probably due to the fact that many selected mutations do not cause amino acid substitutions.

determine unambiguously the nature of the his-3 locus or its product(s). Further genetic analysis seems of doubtful value, especially since there are considerable drawbacks to fine structure mapping in Neurospora. The existence, in Neurospora, of genetic factors which control the frequency of allelic recombination must cast some doubt on the validity of the detail of fine structure maps which have been constructed in this organism (Catcheside, Jessop and Smith, 1964). The existence of such factors acting upon the his-3 locus has now been demonstrated (Jha, 1967; Catcheside, 1968).

Direct evidence that the his-3 locus does not specify three independent enzymes was obtained when Creaser, Bennett and Drysdale (1965) found that mutants lacking pyrophosphohydrolase or cyclohydrolase activities contained histidinol dehydrogenase with altered kinetic properties. Thus partially purified histidinol dehydrogenase from mutants in complementation groups B, D, E and F (Catcheside's data) had different kinetic properties from that of the wild type organism. Clearly, if the his-3 locus specifies three independent enzymes, then a mutation which affects one of those

Unless the mutation results in a reading frame shift, in which case the enzyme is unrecognisable, and cannot be the object of kinetic investigation.

enzymes should have no qualitative effect on another.¹ This evidence was extended when highly purified preparations of histidinol dehydrogenase were obtained (Creaser, Bennett and Drysdale, 1967). It was found that the enzyme from mutant K445 (complementation group F; Catcheside's data) contained a single amino acid replacement when compared with the wild type enzyme (Bennett and Creaser, 1967). Since group F mutants have functional histidinol dehydrogenase, but lack one or both of the other enzymic functions, it was postulated that the purified histidinol dehydrogenase was also responsible for these other enzymic functions. This prediction could not be tested directly because assays for PRATP pyrophosphohydrolase and PRAMP cyclohydrolase were not available.

The examination of the structure and function of histidinol dehydrogenase from his-3 mutants has provided strong evidence that this protein is specified by the entire his-3 locus. It does not follow that his-3 is a single structural gene, because the protein

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Unless the mutation results in a reading frame shift, in which case the enzyme is unrecognisable, and cannot be the object of kinetic investigation.

may consist of more than one polypeptide type. If this is so, then we may ask whether the interaction of these types is obligatory, or whether they may exist as functionally independent molecules under certain conditions. In practice the following questions can be asked.

(1) Can the three activities associated with his-3 be separated, to appear in different fractions during protein purification procedures? If so then are the proteins responsible for these activities different only in their physical properties, or in their chemical and physical properties? How do these proteins interact?

(2) If the three activities are carried by a single protein species does that protein consist of more than one type of polypeptide?

(3) Is the histidinol dehydrogenase which has been purified from Neurospora crassa a trifunctional protein?

It is clear that whatever the answer to these questions, the protein(s) specified by his-3 is sufficiently unusual to be worthy of further study.

CHAPTER 2

Materials and Methods

Where extinction measurements are given they refer to solutions in a 1cm light path. All temperatures are given in centigrade units. Unless otherwise stated, per cent solutions refer to w/v ratios in water.

1 Materials

PRPP.Mg₂.2H₂O was obtained from Mann Research Laboratories, and its purity was given as 85%. A sample was subjected to high voltage paper electrophoresis in 0.02M sodium citrate buffer pH 3.6, at 50v/cm for 20 min at 0°. The paper was dried at 100° to liberate pyrophosphate from the PRPP (Kornberg, Lieberman and Simms, 1955), and the region containing PRPP was identified by staining for acid labile phosphate (Bandurski and Axelrod, 1951), and for reducing sugar (Partridge and Westall, 1948). A PRPP sample of known weight, which had been run in parallel, was eluted from the paper with water. The total phosphate was liberated and assayed by a method to be described, and the PRPP content of the commercial product was estimated to be 75% by this method. Values of PRPP concentration given in the text are weights of the

commercial product corrected by this factor. PRPP was stored as a 2.0mM solution in 3.0mM ethylenediamine-tetra-acetic acid. The solution was brought to pH 7.0 by the addition of a 0.1M tris (free base) solution, and was stored at -15° .

ATP, NAD, 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyl tetrazolium bromide (MTT tetrazolium), and phenazine methosulphate were obtained from Sigma Chemical Company, and 5-amino-4-imidazolecarboxamide riboside (AIC-AR) from California Biochemical Corporation. Orcinol was obtained from British Drug Houses Ltd., and was twice recrystallised from benzene before use. L-histidinol was obtained from Haco S.A., Bern, Switzerland. DNase and RNase were obtained from Worthington Biochemical Corporation.

Sephadex G-15, G-25 G-100 and G-200 were obtained from Pharmacia, Sweden. DEAE, TEAE and CM-celluloses and hydroxylapatite were obtained from BioRad Laboratories.

2 Organisms

Neurospora crassa Emerson a wild type strain was used throughout this work. All growth was at 25° on Vogel's 'N medium' (Vogel, 1955) containing 2% sucrose. The organism was maintained on slopes of the same

medium containing 1.2% Oxoid Ionagar No. 2. Slope cultures were grown for five to seven days, and stored at 4⁰.

Salmonella typhimurium histidine mutants EF-135, E-709 and I-648 were obtained from Dr P. Hartman, Johns Hopkins University, Baltimore, U.S.A. Permanent stocks were prepared by growing the organism in a broth culture, and lyophilising a few drops of the culture adsorbed onto a strip of sterile filter paper. The lyophilised preparations were sealed under vacuum in glass phials. Working stocks were maintained on slopes of 1% Oxoid Nutrient Agar. Stocks were routinely screened for contaminants and revertants before use.

3 Large scale growth of organisms.

(a) Neurospora

The organism was grown in bottles containing 8 litres of minimal medium. Each bottle was inoculated with a conidial suspension obtained by growing the organism on 50ml agar slopes of the same medium. The cultures were aerated by bubbling. After four days the cultures were harvested at 4⁰ using an MSE basket centrifuge of 3 litre capacity lined with Whatman 3MM filter paper. The resulting mycelial pad was broken

up, and lyophilised in a Martin-Christ freeze drier for six days. The dried material was ground to a fine powder using a domestic coffee grinder, and stored desiccated at -15° . The yield of dry powder was 450-500g from 160 litres of culture.

(b) *Salmonella*

The minimal medium used throughout was the 'E medium' of Vogel and Bonner (1956) supplemented with a trace element solution as described by Ames, Garry and Herzenberg (1960). The concentration of D-glucose was 0.5% in all cases. Minimal agar medium was prepared using 1.2% Oxoid Ionagar No. 2. Mutants were initially grown on 2ml slopes of minimal agar containing 2.5×10^{-4} M histidine. A small amount of inoculum was transferred to a bubbled 10ml culture containing 10^{-4} M histidine. After stationary phase had been reached 1ml of this culture was transferred to a second tube containing the same medium, but supplemented with 10^{-4} M histidinol. After 12 hours, 2ml of this culture was used to inoculate 1 litre of minimal medium containing 5×10^{-5} M histidinol. The culture, in a 2l conical flask, was aerated by shaking in a New Brunswick rotary shaker, model G-25. The culture was harvested just before stationary phase ($E_{650\text{mu}}$ 0.9-1.1), after 10-12h

growth, by centrifuging at 5000 x g at 0° for 30min. The pellet was resuspended in 500ml 0.05M tris/HCl, pH 7.5, and centrifuged again. The resulting pellets were stored at -15°. The yield of wet paste was 4-5g/l. The same procedure was used for the growth of the wild type organism except that the medium contained no histidine source.

Histidinol was used as a source of histidine in order to derepress the enzymes of the histidine pathway (see, for example, Loper and Adams, 1965). A lag phase of four to six hours follows the transfer of the organisms from a medium containing histidine to a medium containing histidinol. During this period revertants have a selective advantage, and the final inoculum was therefore screened for ability to grow in the absence of histidine. Mutant EF-135 (a deletion mutant) never reverted, but revertants were occasionally found in cultures of E-709 and I-648. The use of adenine as a growth supplement did not improve the growth rates of these mutants, nor did it improve the levels of histidinol dehydrogenase activity in crude extracts (cf. Smith and Ames, 1964).

4 Preparations of crude extracts of Salmonella

The bacteria (up to 8ml of paste) were sheared using a Hughes Block at -15° . No abrasive was used. The resulting material was allowed to thaw out, and the volume was adjusted to 20ml with 0.05M tris/HCl, pH 7.5. 100ug each of DNase and RNase were added, and the mixture incubated and stirred at 20° . As soon as the mixture had lost its highly viscous properties (usually 2-3min) it was brought to 0° , and centrifuged at 10,000 x g for 30 min. The supernatant was dialysed against two changes of 2l. 0.05M tris/HCl, pH 7.5 for six hours. The extract was then divided into .1ml aliquots and stored at -15° . All preparations were tested for histidinol dehydrogenase activity. Mutant extracts were tested for ability to convert PRPP to AIC-R-P to ensure that all extracts contained an enzymic block in this section of the pathway. Under these conditions of preparation and storage, extracts lost no detectable histidinol dehydrogenase activity over a period of three months.

5 General methods

Unless otherwise stated protein concentration was determined by the method of Lowry et al. (1951), using fraction II human gamma globulin as a standard.

2-mercaptoethanol was found to interfere with this method of estimation. An aqueous solution of 0.01% (v/v) 2-mercaptoethanol gave an $E_{650\text{mu}}$ reading greater than 2.0. Where 2-mercaptoethanol was present in protein preparations and where sufficient protein was available (at least 4mg/ml) estimations were performed according to the method of Gornall, Bardawill and David (1949).

Ribose was estimated by the method described by Ashwell (1957) using 20% of all volumes. Ribose-5-phosphate was used as a standard.

Inorganic phosphate was estimated by the following method¹. 1 ml of 10% ascorbic acid was added to 6ml 0.42% ammonium molybdate in $\text{N-H}_2\text{SO}_4$. 0.7ml of this solution was added to 0.3ml of a test solution containing 0.005 - 0.05 umoles inorganic phosphate. This solution was incubated for one hour at 37° , and the extinction at 820mu was measured against a reagent blank containing no phosphate. Organic

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The ashing procedure and determination of phosphate described here are taken from 'Information Exchange Group No. 7 on nucleic acids and the genetic code' dated 7/22/65. The title is 'The estimation of inorganic phosphate, total phosphate and phosphatases' by B.N. Ames.

phosphate was liberated by the following ashing procedure. To a 0.01 to 0.10ml sample containing 0.005 - 0.05umoles phosphate was added 0.03ml of 10% magnesium nitrate in 95% (v/v) ethanol. The material was dried and ashed over a strong flame until brown fumes ceased to appear. After cooling 0.3ml of 0.5N HCl was added, the tube was capped with a glass marble and heated in a boiling water bath for 15min. The top of the tube was cooled in a stream of air during this process. After cooling, the sample was assayed for inorganic phosphate as described above. KH_2PO_4 was used as a standard for inorganic phosphate, and ribose-5-phosphate as a standard for organic phosphate. 0.025umoles of KH_2PO_4 and ribose-5-phosphate gave $E_{820\text{mu}}$ readings within 5% when subjected to the ashing and assay procedure (mean value obtained was 0.62).

Bratton-Marshall positive compounds (Bratton and Marshall, 1939) were assayed by a method similar to that described by Ames, Martin and Garry (1961). To 0.50ml of a solution containing 0.005 - 0.05umole of diazotisable material was added 0.25ml N-HCl. The solution was brought to 0° , and 0.1ml 5% sodium nitrite added. The solution was allowed to stand for 2min. at 0° . 0.5ml 5% ammonium sulfamate was then

added and the solution allowed to stand for 5min at room temperature. Finally the colour was developed by the addition of 0.4ml 0.1% N-1-naphthylethylenediamine. 2HCl, and the extinction measured at 550mu after standing for 10min. AIC-AR was used as a standard, and 0.025umole gave a reading of 0.37. Compounds which were Bratton-Marshall positive only after mild acid hydrolysis (BBM compounds) were heated for 5min in a boiling water bath after the addition of HCl but before the addition of sodium nitrite in the assay described above. In practice all samples were divided into two, one half being assayed with, and one without acid hydrolysis.

All $MgCl_2$ concentrations given in the text refer to dilutions of the same stock solution. The stock solution was made approximately 0.1M and was then assayed by a method similar to that used by Morrison, O'Sullivan and Ogston (1961). A 20 x 1.5cm column of Zeo-Carb 225 was converted to the hydrogen form by washing with N-HCl, and was then washed with water until the pH rose to 5.5. 20ml of the stock magnesium solution were applied to the column, and the eluate collected as soon as the pH fell below 5.0. The column was washed with water and no further

eluate was collected after the pH had risen above 5.0 (after about 60ml). The total acid eluate was then titrated against standard alkali. The stock solution was found to be 0.096M by this method.

Histidinol dehydrogenase activity was estimated by using the increase in $E_{340\text{mu}}$ which results from the reduction of NAD^+ . Assay conditions were identical to those used by Creaser et al. (1967). The enzyme was incubated with NAD for two minutes in the assay cuvette, and the reaction initiated by the addition of histidinol. The increase in extinction was measured using a Shimadzu automatic recording spectrophotometer. One international unit (I.U.) of enzyme activity is defined as the reduction of μmole of NAD^+ /min. The pH used for this assay is not the optimal pH for histidinol dehydrogenase from S. typhimurium (Loper and Adams, 1965), but, for convenience, has been used here to estimate histidinol dehydrogenase activity both in *Salmonella* and in *Neurospora* extracts.

Column chromatography was always accompanied by fractionation of the eluate using LKB drop counting apparatus. A continuous record of the extinction of the eluate was obtained using LKB 'Uvicord' apparatus. The wavelength of light used was 254mu.

CHAPTER 3

Preliminary observationsIntroduction

In introducing this work it was argued that the kinetic analysis and peptide mapping of histidinol dehydrogenase from his-3 mutants has provided strong evidence that this enzyme is specified by the entire his-3 locus. It follows that this protein should have PRATP pyrophosphohydrolase and PRAMP cyclohydrolase activities, since mutations in the his-3 locus may alter these activities. It seemed logical to begin by investigating this possibility.

Methods

(1) Assay of PRATP pyrophosphohydrolase and PRAMP cyclohydrolase

Estimations of these two activities were made by the mixed extract method described by Ahmed et al. (1964). *Salmonella* histidine mutants used were E-709 (which lacks PRATP pyrophosphohydrolase) and I-648 (which lacks PRAMP cyclohydrolase). Mutant extracts were mixed with the test protein, and the mixture was tested for its ability to convert PRPP to AIC-R-P.

Ability demonstrates the presence in the test protein of the enzyme activity absent from the mutant extract. The reaction mixture contained, in 1ml: tris/HCl pH 8.6, 50umoles; PRPP, 0.4umoles; ATP, 5.0umoles; $MgCl_2$, 15umoles; NH_4Cl , 40umoles; 0.5mg protein from a *Salmonella* mutant and about 1mg of test protein. The mixture was incubated at 34° for 40min, and was then assayed for AIC-R-P. Similar reaction mixtures containing only one of the two protein components were used as controls. The pH of the reaction mixture was initially 8.4, and fell to 8.2 during the course of the incubation.

(2) Preparation of histidinol dehydrogenase

Histidinol dehydrogenase was prepared from *Neurospora crassa* by the method of Creaser et al. (1967). Since frequent references will be made to this purification procedure, the purification table is shown in table 1.3.

Results

The amount of Bratton-Marshall positive material was always the same before and after mild acid hydrolysis, showing that all the BBM material produced was converted to AIC-R-P.

Table 1.3 Purification of Neurospora histidinol dehydrogenase (Table 1 from Creaser et al., 1967).

Step	Procedure	Total Protein (mg)	Total activity (IU)
1	Extraction from dry mycelium	26,100	1200 - 1800
2	Precipitation of nucleic acids	15,800	2180
3	(NH ₄) ₂ SO ₄ fractionation	3,360	1800
4	G100 gel filtration	3,050	780
5	Adsorption-desorption on DEAE-cellulose	960	760
6	TEAE-cellulose chromatography (1)	500	430
7	Recycling G100 gel filtration	80	220
8	TEAE-cellulose chromatography (2)	50	220

The purified histidinol dehydrogenase contained no detectable pyrophosphohydrolase or cyclohydrolase activity as measured by the mixed extract method. However, when material was assayed after each stage of the purification scheme, it was found that after each of steps 1,2,3,4 and 5 the protein contained both activities. Histidinol dehydrogenase negative fractions, normally discarded at each of these steps, contained neither activity. After purification step 6 no pyrophosphohydrolase or cyclohydrolase activity could be detected in the dehydrogenase positive or dehydrogenase negative fractions, and the same result was observed after the remaining purification steps. These results are summarised in table 2.3

Since pyrophosphohydrolase and cyclohydrolase activities could not be detected in any fraction after TEAE-cellulose chromatography (purification step 6), it seemed possible that this procedure resulted in a physical change in histidinol dehydrogenase which destroyed the other two catalytic activities. Attempts were therefore made to reactivate the dehydrogenase active fraction obtained after purification step 6. It was found that if this fraction was dialysed for 12hr at 4⁰ against 0.1M

Table 2.3 Fractionation of PRATP pyrophosphohydrolase and PRAMP cyclohydrolase during the purification of histidinol dehydrogenase.

Purification Step	Ability to complement:	
	I-648 extracts	E-709 extracts
1	++	++
2	+++	+++
3	++++	++++
4	+++	+++
5	++++	++++
6	-	-
7	-	-
8	-	-

Table 3.3 Effect of re-activation with 2-mercaptoethanol

Purification Step	Ability to complement:	
	I-648 extracts	E-709 extracts
6	++	++
7	+++	+++
8	+	+

Dehydrogenase negative fractions did not complement with I-648 or E-709 extracts, either before or after re-activation with 2-mercaptoethanol.

sodium/potassium phosphate buffer pH 6.8 containing 0.2% (v/v) 2-mercaptoethanol, then both pyrophosphohydrolase and cyclohydrolase activities could be detected. Dehydrogenase active fractions could be similarly reactivated after purification steps 7 and 8. Neither activity could be induced in dehydrogenase negative fractions. Pyrophosphohydrolase and cyclohydrolase activities after step 8 were very low. The purified protein had approximately the same specific activity as the crude extract. These results are summarised in Table 3.3

Discussion

It will be noted that a semi-quantitative notation has been used to indicate levels of pyrophosphohydrolase and cyclohydrolase activities. This method has been used because there are a number of reasons why the production of AIC-R-P by a mixed extract may not provide a direct measure of the enzyme activity which is being assayed.

(1) The amount of AIC-R-P produced is measured after 40min. The validity of the assay depends on the production of AIC-R-P being linear over this period. In fact this is not the case. A crude cell extract of *Neurospora* was assayed for pyrophosphohydrolase

activity by the mixed extract method, and samples were taken and assayed for AIC-R-P at 5 min intervals up to 40min. At no time after 10min was the production of AIC-R-P linear with respect to time. Production may have been linear up to 10 min, but the AIC-R-P produced in this time was insufficient to measure accurately.

(2) During the incubation of the mixed extracts, PRPP and some of the intermediates of the histidine pathway may be subjected to spontaneous breakdown, and to attack by enzymes other than those of the histidine pathway (PRPP, PRATP and PRAMP are probably attacked by non-specific phosphatases). Thus competition for substrates exists between histidine pathway enzymes, and those factors causing spontaneous and enzymic degradation. Provided that these factors are constant in all assays, the method gives an accurate measure of the activity of the histidine pathway enzymes. It is clear, however, that these factors are not constant. A crude mycelial extract of *Neurospora* may contain large amounts of phosphatases. An apparent increase in pyrophosphohydrolase activity (as measured by the mixed extract method) as a result of protein fractionation, may be due to an increase in pyrophosphohydrolase activity or to a decrease in phosphatase activity.

(3) AIC-R-P is an intermediate in the biosynthesis of purine nucleotides. The possibility exists that, under the conditions of the mixed extract assay, the AIC-R-P produced may be further metabolised.

It is clear that the assay of pyrophosphohydrolase and cyclohydrolase by the mixed extract method involves a number of variables, the effects of which are difficult to assess. Within the limits of the assay method, however, the results may be summarised as follows.¹

(1) Pyrophosphohydrolase and cyclohydrolase activities exist, or can be induced in dehydrogenase active fractions throughout the purification of histidinol dehydrogenase from Neurospora crassa. The two activities are progressively reduced during the purification.

(2) Neither activity exists, nor can be induced in dehydrogenase negative fractions.

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Similar results to those summarised here have been obtained by Ahmed (1966) using the same assay procedure. He showed that protein fractionation procedures based both on molecular weight and charge differences failed to separate histidinol dehydrogenase, PRATP pyrophosphohydrolase and PRAMP cyclohydrolase activities. He interpreted these observations to mean that the products of the his-3 locus combined to form an enzyme aggregate.

(3) Where one of the two activities exists, the other also exists. No protein fraction was found which contained cyclohydrolase but not pyrophosphohydrolase, or vice versa. Furthermore, both activities exist at approximately the same levels.

These results, taken in combination with those of Ahmed (1966), provide evidence that histidinol dehydrogenase, PRATP pyrophosphohydrolase and PRAMP cyclohydrolase activities reside in a single protein. They also demonstrate, however, that the loss of pyrophosphohydrolase and cyclohydrolase activities need not be accompanied by the loss of histidinol dehydrogenase. Finally, the results demonstrate the limitations of the mixed extract assay technique. A detailed investigation of this problem requires quantitative determinations of pyrophosphohydrolase and cyclohydrolase activities.

CHAPTER 4

Preparation of PRATP, and assay of the overall
reaction PRATP \rightarrow PRAMP \rightarrow BBM II

Introduction

Methods of assaying PRATP pyrophosphohydrolase and PRAMP cyclohydrolase have been described by Smith and Ames (1965). An initial difficulty lies in the production of sufficient quantities of the substrates PRATP and PRAMP, because both intermediates are unstable and their production is expensive and tedious (Ames et al., 1961; Smith and Ames, 1965). The cyclohydrolase reaction, PRAMP \rightarrow BBM II is relatively easy to assay because the reaction is accompanied by a spectral change (Smith and Ames, 1965). The pyrophosphohydrolase reaction, PRATP \rightarrow PRAMP is much more difficult to measure. Two methods were used by Smith and Ames (1965). The first involves the purification and assay of PRAMP produced by reaction mixtures. A single activity estimation requires the chromatography of several samples from a reaction mixture, followed by the identification and assay of the PRAMP from each chromatogram. As a routine assay this is not a practical proposition. The alternative is to

measure the production of pyrophosphate, but this method is also difficult because of the lability of the β and γ phosphate groups of PRATP, and because the assay also measures the action of non-specific phosphatases. Smith and Ames (1965) reported that neither assay method was very satisfactory.

The results described in chapter 3, and those of Ahmed (1966), suggest that, in *Neurospora*, PRATP pyrophosphohydrolase and PRAMP cyclohydrolase cannot be separated. This suggested that it might be feasible to measure the overall reaction $\text{PRATP} \rightarrow \text{PRAMP} \rightarrow \text{BBM II}$, thus avoiding the difficulties involved in the assay of the reaction $\text{PRATP} \rightarrow \text{PRAMP}$. The mixed extract assay method could be used at the same time to ensure that any protein preparation unable to catalyse the overall reaction could not catalyse either of the reactions singly.

Preparation of PRATP

PRATP was prepared and purified by methods similar to those described by Ames et al. (1961). A number of differences exist, however, and the preparation will be described in full.

The incubation mixture for the generation of PRATP contained, in 30ml 0.05M tris/HCl, pH 8.6: 12umoles

PRPP; 150umoles ATP; 450umoles MgCl_2 , and 15mg of protein from an extract of Salmonella typhimurium EF-135 (a deletion mutant which lacks PRATP pyrophosphohydrolase and PRAMP cyclohydrolase). The mixture was incubated at 37° . The production of PRATP is accompanied by an increase in extinction at 290mu, and the measurement of this increase provides an assay for the reaction (Ames et al., 1961; Voll, Appella and Martin, 1967). For the purpose of following the production of PRATP in the generation mixture described above, this assay method was found unsatisfactory because of the very high extinction value of the mixture at zero time. The production of PRATP was followed by removing 1ml samples at 5min intervals, and adding 20umoles NH_4Cl , and a cell free extract of wild type Salmonella, containing 0.5mg of protein. The sample was then incubated again for 30 min, and assayed for AIC-R-P. To prevent further production of PRATP during this second incubation, the sample was made 0.5mM with respect to histidine (Ames et al., 1961; Martin, 1963b). In a trial run the amount of AIC-R-P produced by the generation mixture was found to be at a maximum after 40 min.

analysis: 0.1umole of PRATP (determined

After 40 min the mixture was diluted to 100ml with water and brought to pH 6.5 with N-HCl. After this stage all procedures were carried out at 4°. The solution was applied to a 100 x 1.2cm column of DEAE-cellulose, previously equilibrated with 0.02M KCl, and the column was eluted with a 0.02 - 0.5M linear gradient of KCl of total volume 600ml. PRATP was identified by its spectrum, and by its conversion to AIC-R-P. PRATP was only partially separated from ADP, as shown in fig. 1.4. The fractions containing PRATP were lyophilised, and the dried material dissolved in 1ml of water. This solution was then chromatographed on a 30 x 3cm column of Sephadex G-15, previously equilibrated with water. This removes the KCl, and, as shown in fig. 2.4, achieves further removal of ADP. The PRATP was lyophilised and stored at -15° either as the dry material, or as a 3mM solution at pH 6.0 in 0.02M sodium citrate buffer. The yield was 4.5umoles. This material was used for assay purposes, but was contaminated with ADP. A small amount of this preparation was rechromatographed on DEAE-cellulose to remove all the ADP, and this repurified material was subjected to the following analysis: 0.1umole of PRATP (determined

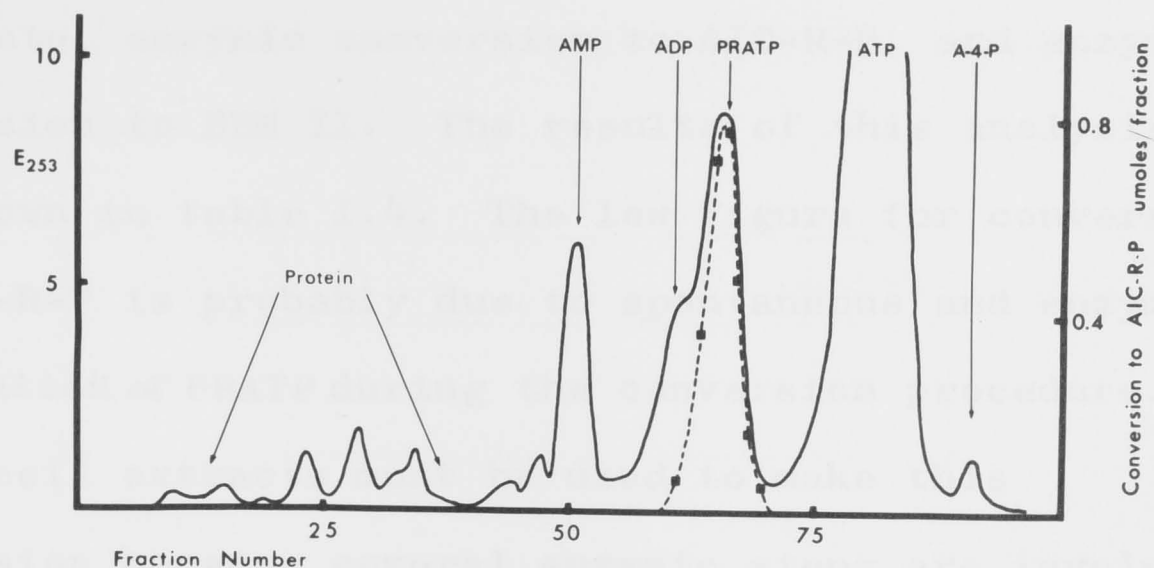


Figure 1.4 Chromatography of PRATP on DEAE-cellulose.

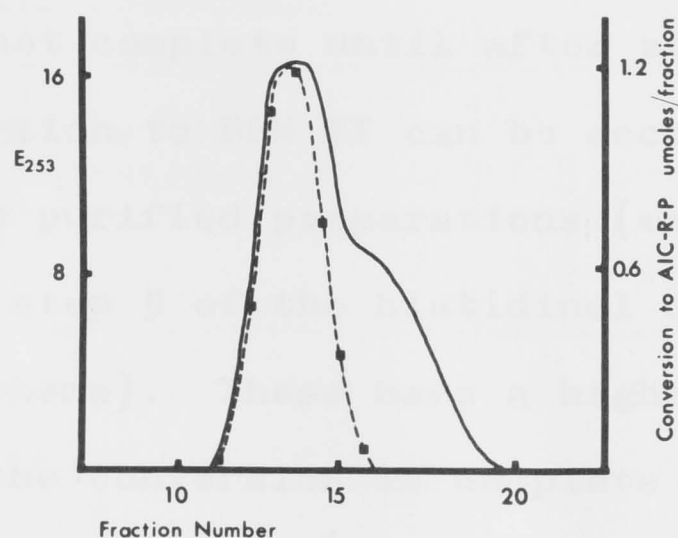


Figure 2.4 Chromatography of PRATP on Sephadex G15.

Figs. 1.4 and 2.4: purification of PRATP. A preparation of PRATP was applied to a 100 x 1.2cm column of DEAE-cellulose, and the chromatogram developed with a 0.02 - 0.5M linear gradient of KCl. The fractions containing PRATP were lyophilised, dissolved in 1ml of water and applied to a 30 x 3cm column of Sephadex G15 previously equilibrated with water. The column was then washed with water. The eluate from both chromatograms was monitored (—), and the fractions were assayed for AIC-R-P after incubation with a wild type Salmonella extract (■-----■).

spectrophotometrically) was assayed for ribose, total phosphate, enzymic conversion to AIC-R-P, and enzymic conversion to BBM II. The results of this analysis are shown in table 1.4. The low figure for conversion to AIC-R-P is probably due to spontaneous and enzymic degradation of PRATP during the conversion procedure. Crude cell extracts must be used to make this conversion because several enzymic steps are involved. These extracts have a low specific activity, and the conversion is not complete until after about 30 min at 37⁰. Conversion to BBM II can be accomplished using partially purified preparations (material obtained after step 5 of the histidinol dehydrogenase purification scheme). These have a higher specific activity, and the conversion is complete in about 5min.

The repurified PRATP was used in the standard PRATP → BBM II assay to be described. It was found that ADP, at 50% of the substrate concentration, had no effect on the observed reaction rate, and it was therefore decided that complete removal of ADP from PRATP preparations was unnecessary for assay purposes (the ADP content of PRATP preparations used for assay purposes was found to be 15-20%). This estimate was

Table 1.4 Analysis of PRATP preparation.

The values are for 0.1umole PRATP determined spectrophotometrically.

TEST	umoles
Total phosphate	0.37
Ribose	0.21
AIC-R-P by enzymic conversion	0.078
BBM II* by enzymic conversion	0.095 ^a 0.093 ^b

* Conversion to BBM II was estimated by two methods:

- (a) increase in absorbancy at 290mu
- (b) Bratton-Marshall test after acid hydrolysis.

obtained by a comparison of the U.V. spectrum with the PRATP content as estimated by conversion to BBM II.).

Assay of the overall reaction PRATP \rightarrow PRAMP \rightarrow BBM II

The conversion of PRATP to BBM II was followed by measuring the increase in extinction at 290mu which results from the hydrolysis of the 1-6 bond of the adenine ring. The conversion of a 1mM solution of PRATP to BBM II results in an increase in extinction of 4.4 units at pH 8.5 (Smith and Ames, 1965). The assay was performed in a 1cm path length cuvette of 4mm path width, and the cuvette was maintained at 37⁰ in a water-jacketed cuvette housing. Increase in extinction was measured using a Shimadzu recording spectrophotometer. The assay solution contained, in 1ml 0.05M tris/HCl pH 8.5: 5umoles 2-mercaptoethanol; 2umoles MgCl₂; 0.15umoles PRATP, and enzyme. Buffer containing 2-mercaptoethanol and MgCl₂ was preincubated at 37⁰. Buffer and enzyme were incubated together for 2 min in the assay cuvette, and the reaction was initiated by the addition of 0.05ml of the PRATP solution. A reaction blank contained no substrate.

The concentration of magnesium used in this assay is an order of magnitude lower than that required for enzyme PRATP pyrophosphohydrolase from Salmonella

(Smith and Ames, 1965). Fig. 3.4 shows a double reciprocal plot (Lineweaver and Burk, 1934) of reaction velocity versus magnesium concentration over the range $2 \times 10^{-3} - 1 \times 10^{-4} \text{M.}^1$ The K_m for magnesium is approximately 10^{-4}M. No detectable increase in reaction velocity could be achieved by using MgCl_2 concentrations higher than 2.0mM. At concentrations above 4.0mM protein began to precipitate during the assay, and the resulting turbidity interfered with the photometric measurements.

Under the conditions of assay described, the reaction velocity is linear during the first 30sec, and is proportional to enzyme concentration over the range 8×10^{-3} to $1 \times 10^{-1} \text{ I.U.s/ml}$ in the assay cuvette (where one I.U. is defined as the conversion of 1umole PRATP to BBM II per min). Above and below these limits the observed reaction rates fall below the values obtained by extrapolating the linear portion of the relationship. Fig. 4.4 shows the effect of enzyme concentration on the observed reaction rate. All activity levels quoted in this work have been derived

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The results illustrated in figs. 3.4 and 4.4 were obtained using the purified protein preparation described in Chapter 6.

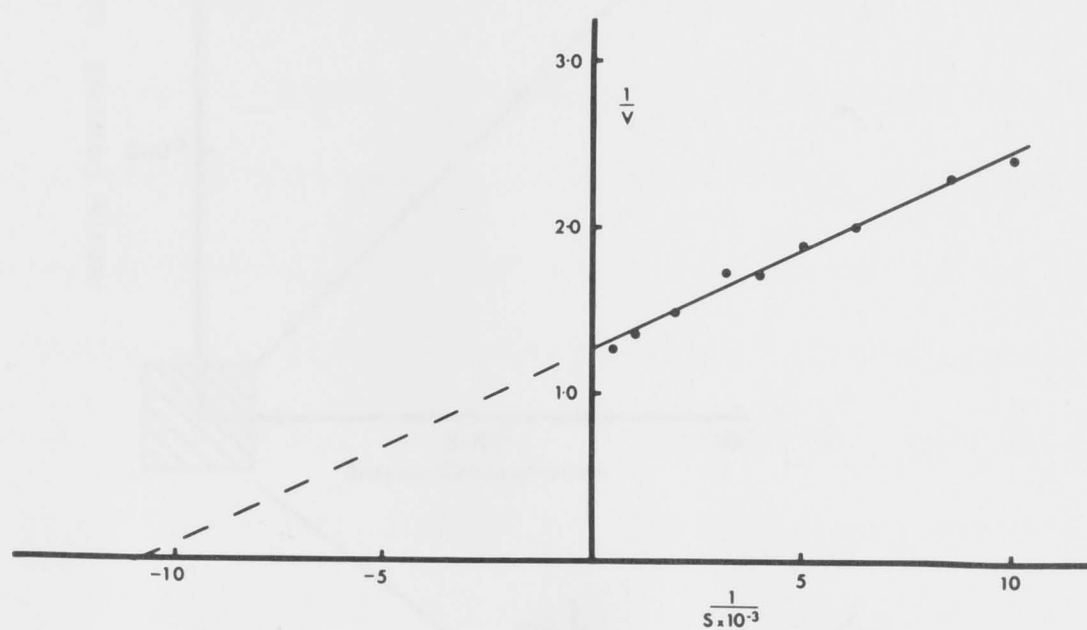


Figure 3.4 Double reciprocal plot of magnesium concentration versus reaction velocity.

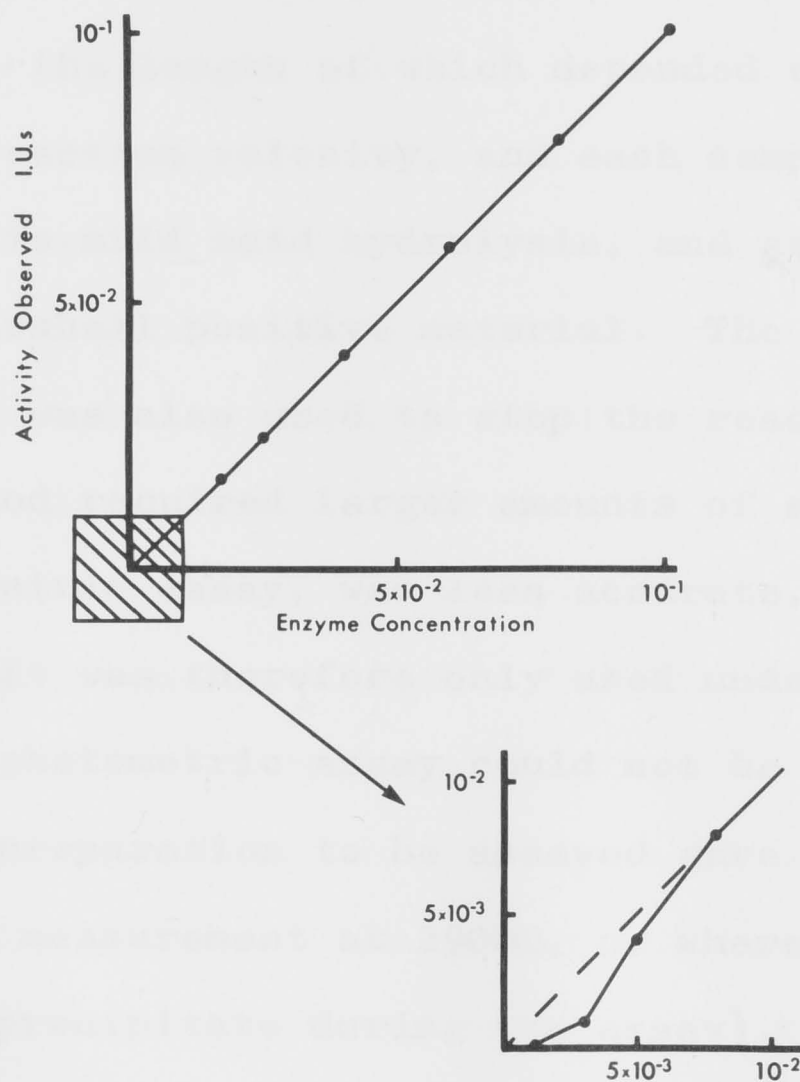


Figure 4.4 Relationship between enzyme concentration and reaction velocity

Conditions of assay are as described in the text. The enzyme concentration is based on the dilution of a solution containing 10^{-1} I.U./0.1ml

from assays in which the observed reaction velocities fell in the linear part of this plot.

An alternative assay procedure was sometimes used. The concentration of all components was the same, but the volume was 3ml. 0.5ml samples were removed at intervals, the length of which depended on the expected reaction velocity, and each sample was subjected to mild acid hydrolysis, and assayed for Bratton-Marshall positive material. The acid used for hydrolysis was also used to stop the reaction. This assay method required larger amounts of substrate than the photometric assay, was less accurate, and more tedious. It was therefore only used under conditions where the photometric assay could not be used (e.g. where the preparation to be assayed gave a very high extinction measurement at 290mu, or where protein tended to precipitate during the assay).

Three compounds in the histidine pathway respond to the Bratton-Marshall test only after acid hydrolysis: BBM I, II, and III (Smith and Ames, 1964, 1965 and see fig. 2.1). All three intermediates have the same extinction at 290mu. Hence, neither of the two assays described can distinguish between these three intermediates. BBM III is an Amadori rearrangement of

BBM II, and the conversion of BBM II to BBM III in the reaction mixture is irrelevant to the measurement of the reaction $\text{PRATP} \rightarrow \text{BBM II}$. BBM I, however, results from the cleavage of the adenine ring prior to the removal of pyrophosphate. In *Salmonella* the compound is not part of the histidine pathway, but is apparently a product of non-specific activity of PRAMP cyclohydrolase on PRATP (Smith and Ames, 1965). The assay methods which are described here make no distinction between the following reaction sequences:



Thus the assays measure either (a) the consecutive actions of PRATP pyrophosphohydrolase and PRAMP cyclohydrolase, or (b) the non-specific action of cyclohydrolase alone. The possibility that the non-specific action of cyclohydrolase might be being measured can be ruled out by the following observations:

(1) When magnesium is absent from the reaction mixture, the reaction rate observed is less than 5% of that observed in its presence. PRAMP cyclohydrolase from *Salmonella* does not require magnesium, and there is no reason to believe that the *Neurospora* enzyme does so. It seems, therefore, that *Neurospora*

cyclohydrolase acts on PRATP with less than 5% the efficiency with which it acts upon PRAMP, under the conditions of assay.

(2) The products of the assay can be converted to AIC-R-P using a *Salmonella* extract blocked in pyrophosphohydrolase (E-709). BBM II is converted to AIC-R-P, while BBM I is not a substrate for any of the histidine pathway enzymes in *Salmonella* (Smith and Ames, 1965). Whenever this test was applied to the reaction products of a photometric assay, the AIC-R-P produced was equal to the calculated amount of BBM II produced during the assay.

The assay methods used were therefore considered to provide valid measurements of the overall reaction $\text{PRATP} \rightarrow \text{PRAMP} \rightarrow \text{BBM II}$.

It is convenient to state here that during the work described in this, and in subsequent chapters, no protein preparation was found which contained pyrophosphohydrolase but not cyclohydrolase activities, or *vice versa*. Thus any preparation which failed to convert PRATP to BBM II also failed to complement E-709 or I-648 extracts in the conversion of PRAMP to AIC-R-P.

Table 1.3 shows the effect of the purification of histidinol dehydrogenase on the ability of the protein

CHAPTER 5

Experiments with histidinol dehydrogenaseIntroduction

The experiments described in Chapter 3 demonstrated the co-fractionation of histidinol dehydrogenase, PRATP pyrophosphohydrolase and PRAMP cyclohydrolase. It was apparent, however, that the latter two activities were considerably reduced by some of the fractionation procedures used. The use of the quantitative assay procedure described in Chapter 4 allows a more precise investigation of these phenomena.

Results

It is convenient to state here that during the work described in this, and in subsequent chapters, no protein preparation was found which contained pyrophosphohydrolase but not cyclohydrolase activities, or vice versa. Thus any preparation which failed to convert PRATP to BBM II also failed to complement E-709 or I-648 extracts in the conversion of PRPP to AIC-R-P.

Table 1.5 shows the effect of the purification of histidinol dehydrogenase on the ability of the protein

Table 1.5 Purification of histidinol dehydrogenase:
ability of dehydrogenase active fractions
to catalyse the reaction PRATP \rightarrow BBM II

Purification Step	Total enzyme activity (I.U.)	
	histidinol dehydrogenase	PRATP \rightarrow BBM II
1	≤ 750	690
2	600	550
3	600	500
4	390	125
5	275	55
6	230	10
7	85	7
8	65	0.6

The starting material was about 200gm dry mycelium
Protein obtained after steps 6, 7 and 8 was
re-activated with 2-mercaptoethanol as previously
described. Dehydrogenase negative fractions could
not convert PRATP to BBM II, either before or
after re-activation

The problems involved in the measurement of
histidinol dehydrogenase activity in crude extracts
have severely hampered attempts to demonstrate repression
or derepression of this enzyme in *Neurospora crassa*.
Purification of the extracts allows a more accurate
estimation of activity, but the validity of the results
then depends upon the reproducibility of the
purification procedures.

to catalyse the reaction $\text{PRATP} \rightarrow \text{BBM II}$. All preparations after stage 6 of the purification have been reactivated with 2-mercaptoethanol by the method previously described. Several points need to be made about this table.

(1) A maximum estimate only has been given for the histidinol dehydrogenase activity of the crude extract. Histidinol dehydrogenase activity is very difficult to measure at this stage because the crude extract rapidly reduced NAD^+ in the absence of histidinol.¹ The hydrogen donor in this system is unknown, but it cannot be removed by dialysis or by gel filtration. This endogenous activity towards NAD^+ is partially removed during purification steps 2, 3 and 4, but is not completely removed until after step 5. Only in the crude extract, however, is the reduction of NAD^+ in the absence of histidinol so great as to swamp any increase in reduction rate brought about by its addition. The maximum value of 750 I.U.s was obtained by estimating

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The problems involved in the measurement of histidinol dehydrogenase activity in crude extracts have severely hampered attempts to demonstrate repression or derepression of this enzyme in Neurospora crassa. Purification of the extracts allows a more accurate estimation of activity, but the validity of the results then depends upon the reproducibility of the purification procedures.

the minimum activity which could have been detected in the presence of the endogenous activity found in the crude extract. Histidinol dehydrogenase activity is also difficult to measure after steps 2, 3 and 4 for the same reason, and because the protein after these stages is contained in large volumes of buffer. The activity per unit volume is therefore very low.

(2) It is clear that during the purification the ability to catalyse the reaction $\text{PRATP} \rightarrow \text{BBM II}$ is progressively reduced, relative to histidinol dehydrogenase activity. The largest losses of activity occur during purification steps 6 and 8, and two TEAE cellulose fractionations, and the specific activity of the purified protein towards PRATP is only slightly more than that of the crude extract. The purified protein converts PRATP to BBM II at only 1% the rate at which it reduces NAD^+ in the presence of histidinol.

(3) The results given in Table 1.5 are not entirely reproducible. In a small number of cases the protein obtained after purification step 6 could not be reactivated with 2-mercaptoethanol, and the ability to convert PRATP to BBM II was irreversibly lost. Yet these preparations, extraordinary in this respect,

were ordinary in all other respects (e.g. total protein and total dehydrogenase activity recovered). Thus no other factor could be correlated with this loss of pyrophosphohydrolase and cyclohydrolase activity.

These results are unsatisfactory, for although the purified histidinol dehydrogenase can be induced to catalyse the reaction $\text{PRATP} \rightarrow \text{BBM II}$, about 99% of this activity is lost during the purification relative to histidinol dehydrogenase activity. If pyrophosphohydrolase and cyclohydrolase activities are carried by the same molecule as histidinol dehydrogenase activity, then the ratio of the activities should be the same throughout the purification scheme. In fact the ratio is about 1:1 after each of the first three purification steps, and about 100:1 (histidinol dehydrogenase : $\text{PRATP} \rightarrow \text{BBM II}$) in the purified protein.

The action of 2-mercaptoethanol is presumably to effect a physical change in a protein so that the protein recovers the competence to catalyse the reaction $\text{PRATP} \rightarrow \text{BBM II}$, this competence having been lost as a result of the purification procedures. Subjection of purified preparations of histidinol dehydrogenase to treatment with 2-mercaptoethanol

results in only a 1% recovery of this competence relative to histidinol dehydrogenase activity. This low recovery is open to several interpretations.

(1) That purified preparations of histidinol dehydrogenase contain a very minor component which is capable of catalysing the reaction $\text{PRATP} \rightarrow \text{BBM II}$ after treatment with 2-mercaptoethanol.

(2) That histidinol dehydrogenase molecules in the purified preparation are themselves potentially able to catalyse the reaction $\text{PRATP} \rightarrow \text{BBM II}$, but that treatment with 2-mercaptoethanol results in the realisation of this potential in only a small proportion (about 1%) of the molecules (i.e. that treatment sets up an equilibrium which strongly favours the uni-functional form of the molecule).

(3) A third possibility arises from a combination of (1) and (2). Competence to catalyse the reaction $\text{PRATP} \rightarrow \text{BBM II}$ might depend on the interaction of histidinol dehydrogenase with another protein molecule, this interaction being stimulated by treatment with 2-mercaptoethanol. If this second protein alone had no catalytic activity towards PRATP, then its removal during the purification would pass unnoticed. The purified preparation might contain only a very small

amount of this second protein, and this would account for the reactivation of only a small proportion of the histidinol dehydrogenase molecules. If this interpretation were valid, then where a protein fractionation procedure results in a considerable loss of pyrophosphohydrolase and cyclohydrolase activity, remixing of the fractions, followed by reactivation with 2-mercaptoethanol, should result in a much higher recovery of activity. This is not the case. After step 6 of the purification scheme (TEAE-cellulose chromatography), mixing of the fractions did not improve the yield of pyrophosphohydrolase and cyclohydrolase activities obtained by reactivating the histidinol dehydrogenase active fraction alone. Evidence against interpretation (1) can be obtained through similar reasoning. If the activity towards PRATP of purified histidinol dehydrogenase preparations is due to the presence of a minor impurity of independent action, then losses of activity during the purification are presumably due to the partial removal of this component. However, pyrophosphohydrolase and cyclohydrolase activities were never found, nor could they be induced, in dehydrogenase negative fractions. Thus, interpretations (1) and (3) are probably incorrect.

It is significant that major losses of pyrophosphohydrolase and cyclohydrolase activities do not occur until step 6 of the histidinol dehydrogenase purification scheme. Creaser et al. (1965) predicted that histidinol dehydrogenase was specified by the entire his-3 locus, and was therefore a tri-functional protein. This prediction was based on the observation that his-3 mutants which lacked pyrophosphohydrolase and cyclohydrolase, contained histidinol dehydrogenase with altered kinetic parameters. The kinetic measurements were made using proteins purified to stage 5 of the purification scheme. The experiments described in this chapter, however, show that preparations of histidinol dehydrogenase from the 'wild type' organism contain cyclohydrolase and pyrophosphohydrolase activities after purification step 5, but lose these activities after further purification. It follows that interpretations of the kinetic results obtained by Creaser et al. (1965) cannot be extrapolated to more highly purified preparations.

* * * * *

Alternative methods of reactivation

On the assumption that the action of 2-mercaptoethanol is to change the physical properties

of histidinol dehydrogenase molecules such as to restore pyrophosphohydrolase and cyclohydrolase activities, alternative methods were investigated in an attempt to improve the efficiency of this reactivation. These methods generally involved the reduction of di-sulphide linkages, or techniques thought to result in the dissociation and association of protein sub-units. In all cases the treatments were carried out in 0.1M sodium/potassium phosphate buffer, pH 6.8. Protein concentrations between 1 and 2mg/ml were used, and all treatments were performed on purified histidinol dehydrogenase preparations.

(1) The usual reactivation procedure was used with dithiothreitol or reduced glutathione instead of 2-mercaptoethanol. Dithiothreitol was used at concentrations of 50uM to 1.0mM, and glutathione at concentrations of 1.0mM to 25mM. Dithiothreitol was ineffective in restoring pyrophosphohydrolase or cyclohydrolase activities, and at the higher concentrations (0.25 - 1.0mM) the treatment resulted in loss of histidinol dehydrogenase activity. Treatment with glutathione at the higher concentrations resulted in some reactivation of the protein, but was less effective than treatment with 2-mercaptoethanol at the same concentration.

(2) The reduction procedures described in (1) were combined with gentle heat treatment. In these experiments the protein was not dialysed against the reagent, but the reagent added to give the concentrations quoted above. Protein solutions were then heated for 5 or 20 min at 30 or 50°. All results were negative.

(3) Protein solutions in 0.1M sodium/potassium phosphate buffer containing various concentrations of KCl (0.05, 0.10, 0.50 and 1.0M) were allowed to slowly freeze and thaw. This treatment is thought to cause dissociation and re-association of protein subunits (Markert, 1963). All results were negative.

(4) A final method which was attempted, was to subject the purified preparation of histidinol dehydrogenase to those steps in the purification scheme which did not result in losses of pyrophosphohydrolase or cyclohydrolase activity. It was hoped that this treatment might restore the protein molecules to the physical form in which they normally existed in the partially purified extract. The protein was subjected to precipitation with ammonium sulphate in the presence of 0.05M MnCl_2 , followed by Sephadex G100 gel filtration, and adsorption - desorption chromatography on DEAE-cellulose. After each

and all three of these procedures the preparation contained no detectable pyrophosphohydrolase or cyclohydrolase activity.

It is apparent that this is by no means a comprehensive attempt to alter the tertiary or quaternary structure of this protein. It will be appreciated, however, that these experiments consume considerable amounts of purified histidinol dehydrogenase, and of the substrate PRATP. The production of enzyme and substrate places a practical limit on the number of experiments of this kind which can be performed. Furthermore, the trial and error nature of the experiments, together with totally negative results, tends to be somewhat discouraging to the experimenter.

Isolation by DCA * * * * *

Attempts to manipulate the histidinol dehydrogenase purification scheme

The failure of attempts to reactivate the purified protein suggested the alternative approach of manipulating the purification scheme in such a way as to reduce the losses of pyrophosphohydrolase and cyclohydrolase activities. Since 2-mercaptoethanol was partially effective in reactivating the protein obtained after steps 6, 7 and 8 of the purification scheme, it

was decided to equilibrate the chromatography columns used in these steps with 2-mercaptoethanol. Step 4 of the purification scheme was also a cause of loss of activity, so this step was not used. The following procedure was used to prepare material for TEAE-cellulose chromatography (step 6). The starting material was 20 gm of dry mycelial powder.

Steps 1, 2 and 3 of the purification scheme were used unchanged.

Step 4 was replaced by gel filtration of the protein on a 30 x 2cm Sephadex G25 column, previously equilibrated with 0.01M sodium/potassium phosphate buffer, pH 6.8. This resulted in the rapid removal of ammonium sulphate, and prepared the protein for adsorption by DEAE-cellulose. No losses of activity resulted.

Step 5 of the purification (DEAE-cellulose chromatography) was used unchanged.

The solution resulting from step 5 was divided into two equal volumes, and treated as follows. Solution (a) was dialysed against 0.01M sodium/potassium phosphate buffer pH 6.8 according to the usual purification procedure. Solution (b) was subjected to gel filtration on a 30 x 5cm Sephadex G25 column, previously

equilibrated with 0.01M sodium/potassium phosphate, pH 6.8 containing 0.2% (v/v) 2-mercaptoethanol. The alternative of dialysing the protein against phosphate buffer containing 2-mercaptoethanol resulted in precipitation of protein, and considerable losses of all three activities.

Preparation (a) was then chromatographed on TEAE-cellulose and Sephadex G100 (i.e. steps 6 and 7 of the usual purification scheme). Preparation (b) was similarly treated, but in this case both columns were equilibrated with buffers containing 0.2% 2-mercaptoethanol, and eluting buffers also contained 0.2% 2-mercaptoethanol.

The results of these chromatograms are illustrated in figs. 1.5 to 4.5. Chromatography of preparation (a) results in a single zone of histidinol dehydrogenase activity on TEAE-cellulose and Sephadex G100. None of the fractions of either chromatogram is able to catalyse the conversion of PRATP to BBM II (unless they are first reactivated with 2-mercaptoethanol). These results are as predicted by the histidinol dehydrogenase purification scheme. The use of 2-mercaptoethanol has a marked effect, as shown by the chromatography of preparation (b). TEAE-cellulose chromatography results in two zones of

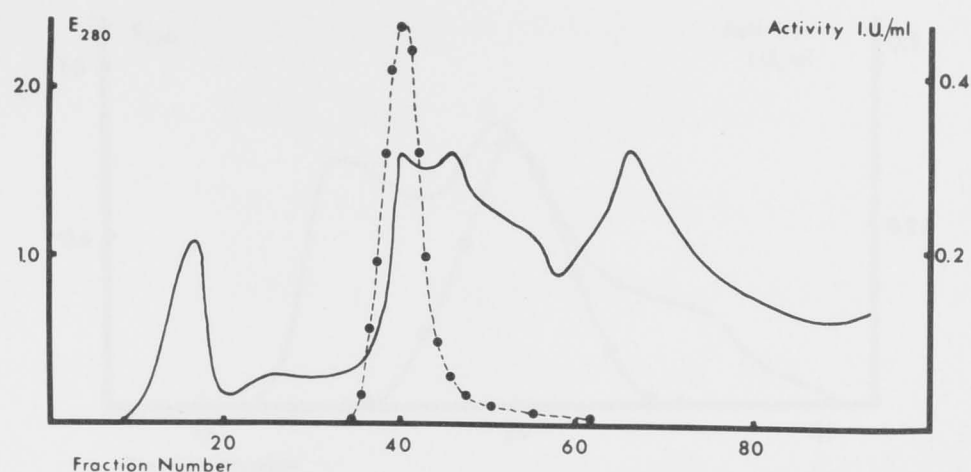


Figure 1.5 Chromatography of histidinol dehydrogenase on TEAE-cellulose

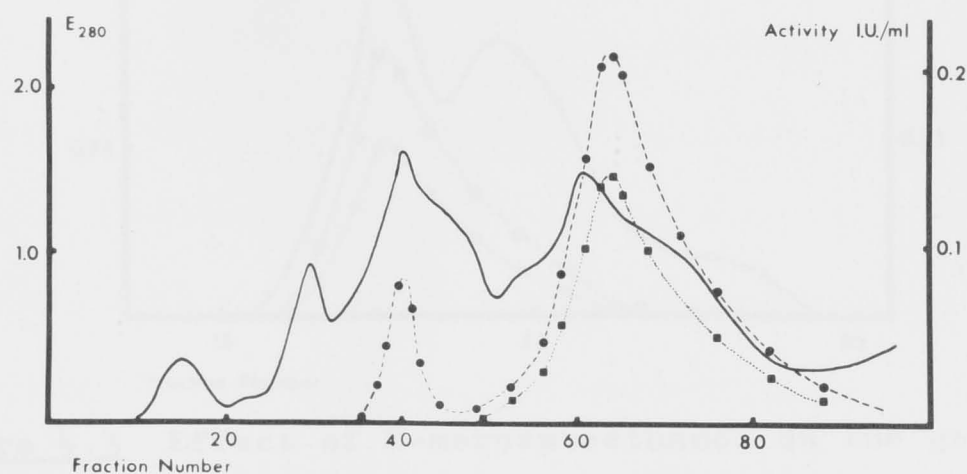


Figure 2.5 Effect of 2-mercaptoethanol on the chromatography of histidinol dehydrogenase on TEAE-cellulose.

The sample was applied to a 40 x 1.4cm column of TEAE-cellulose previously equilibrated with 0.01M sodium/potassium phosphate, pH 6.8. The protein was then eluted with a gradient of increasing phosphate molarity, as described by Creaser *et al* (1967). The eluate was monitored (—), and the fractions assayed for histidinol dehydrogenase activity (•-----•) and for ability to convert PRATP to BBM II (■.....■).

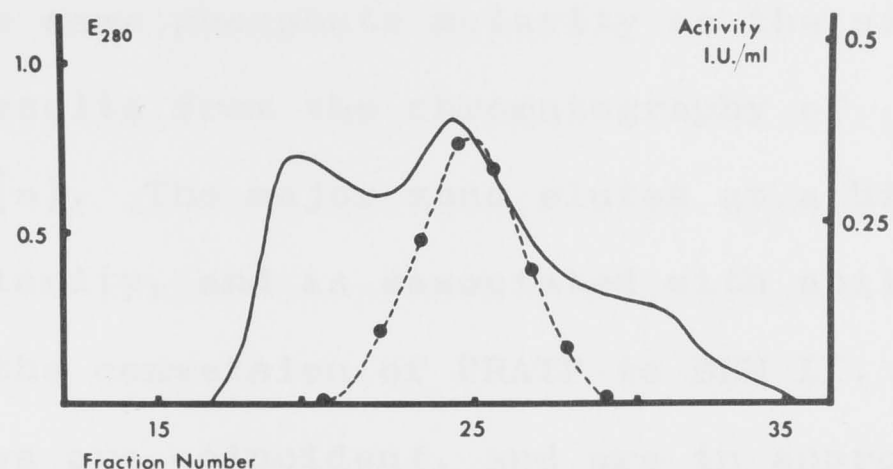


Figure 3.5 Sephadex G100 gel filtration of histidinol dehydrogenase

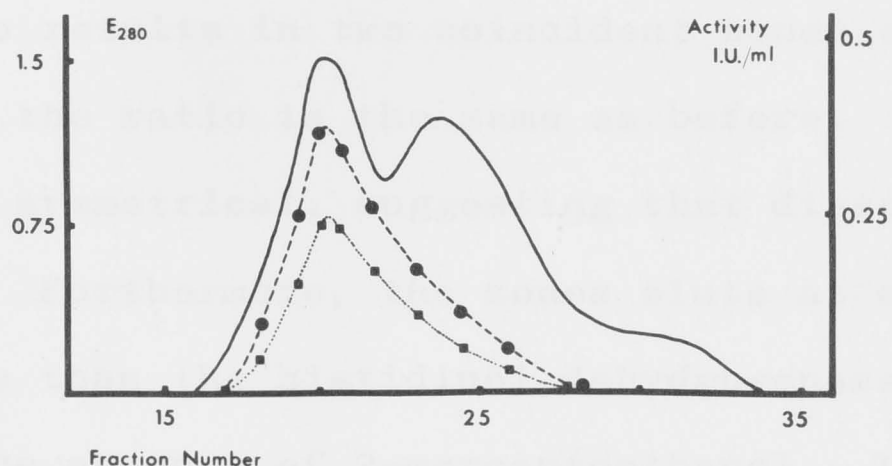


Figure 4.5 Effect of 2-mercaptoethanol on the gel filtration of histidinol dehydrogenase.

Histidinol dehydrogenase active fractions from the chromatograms shown in figs. 1.5 and 2.5 were reduced in volume and subjected to gel filtration on a 45 x 1.5cm column of Sephadex G100 previously equilibrated with 0.1M phosphate buffer, pH 6.8. The protein was eluted with the same buffer at a flow rate of 10ml/hr. The eluate was monitored (—) and the fractions assayed for histidinol dehydrogenase activity (•-----•) and for ability to convert PRATP to BBM II (■.....■)

histidinol dehydrogenase activity. The minor zone elutes at the same phosphate molarity as the single zone which results from the chromatography of preparation (a). The major zone elutes at a higher phosphate molarity, and is associated with ability to catalyse the conversion of PRATP to BBM II. These two activities are coincident, and are in approximately the same ratio as is found in the crude extract. Gel filtration of the major histidinol dehydrogenase component also results in two coincident zones of activity, and the ratio is the same as before. The zones are not symmetrical, suggesting that dissociation is occurring. Furthermore, the zones elute at a lower elution volume than the histidinol dehydrogenase zone obtained in the absence of 2-mercaptoethanol. The effect of 2-mercaptoethanol is thus to retain pyrophosphohydrolase and cyclohydrolase activities, and to increase the molecular weight of histidinol dehydrogenase. The apparent increase in molecular weight due to the presence of 2-mercaptoethanol is a surprising result, since the presence of excess SH groups should decrease the probability of disulphide linkages between polypeptide chains (as well as within

Sephadex G100 or G200 was unsatisfactory because of

them), so that if any change in molecular weight occurred a decrease would be expected.

The ability of 2-mercaptoethanol to stabilise pyrophosphohydrolase and cyclohydrolase activities during these purification procedures suggested that it might be possible to totally purify histidinol dehydrogenase in a trifunctional form by the inclusion of 2-mercaptoethanol in all stages of the purification. The purification procedure of Creaser et al. (1967) was found to be inadequate for this purpose, however, because one of the effects of 2-mercaptoethanol was to alter the elution positions of histidinol dehydrogenase on TEAE-cellulose and Sephadex G100 chromatograms. A different set of impurities therefore remain to be removed after these two fractionations. The inclusion of 2-mercaptoethanol at all stages of the purification scheme results in a preparation containing at least five protein components. Purification of the trifunctional form of the enzyme is therefore a new problem.

Initial attempts to purify the tri-functional protein involved further fractionation of the TEAE-cellulose eluate described above. Gel filtration on Sephadex G100 or G200 was unsatisfactory because of

assymetry of the activity zones. On both gels, however, the zones of activity are coincident. Many procedures were discarded because they resulted in heavy losses of one of the activities. The use of DEAE and TEAE celluloscs at pH 8.0 - 8.5 in tris/HCl buffers was unsatisfactory because in the presence of 2-mercaptoethanol a 95% loss of histidinol dehydrogenase activity occurred, while the eluted protein had no activity towards PRATP if 2-mercaptoethanol was not included.¹ CM-cellulose would not adsorb the proteins in the preparation even at phosphate concentrations as low as 1mM.

The experiments described in this chapter were, in general, unsuccessful. None of the procedures described resulted in a pure protein able to catalyse the pyrophosphohydrolase, cyclohydrolase and dehydrogenase reactions at high rates. Reactivation

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This is a remarkable result in that dialysis of the protein against the same tris/HCl buffers in the presence or absence of 2-mercaptoethanol has no effect on either activity. This suggests that DEAE or TEAE celluloscs play some part in the inactivation of the protein under these conditions. But, as will be seen in Chapter 6, the same effect occurs when sucrose density gradient centrifugation or starch gel electrophoresis are the fractionating techniques used in the presence of tris/HCl buffers.

of the purified histidinol dehydrogenase preparation resulted in some recovery of pyrophosphohydrolase and cyclohydrolase activity, but the recovery was so low that the result could be interpreted in a number of ways. These interpretations could not be tested directly because the levels of heterogeneity predicted by the interpretations were so low as to be undetectable by any physical means. Nevertheless, the experiments described here provide strong evidence that the three enzymic activities are carried by a single protein.

(a) Preparation

The purification finally devised employs eight steps which are summarized in fig. 1.6. The results of these steps are summarized in table 1.6. The starting material was 200gm dry mycelial powder. Unless otherwise stated, all procedures were performed at 4°. 'Phosphate buffer, pH 6.8' refers to an equimolar mixture of NaH_2PO_4 and K_2HPO_4 .

CHAPTER 6

Purification of tri-functional histidinol dehydrogenaseIntroduction

The previous chapter described unsuccessful attempts to reactivate histidinol dehydrogenase, and to manipulate the purification scheme described by Creaser et al. (1967). It was clear from the results of these attempts that a substantially different purification method would be required to obtain a pure protein with all three catalytic activities. Such a purification procedure is described in this chapter. The tri-functional form of the enzyme will be referred to as PRAMP cyclohydrolase to distinguish it from the uni-functional form, histidinol dehydrogenase, as purified by the method of Creaser et al. (1967).

(a) Preparation

The purification finally devised employs eight steps which are summarised in fig. 1.6. The results of these steps are summarised in table 1.6. The starting material was 200gm dry mycelial powder. Unless otherwise stated, all procedures were performed at 4°. 'Phosphate buffer, pH 6.8' refers to an equimolar mixture of NaH_2PO_4 and K_2HPO_4 .

Figure 1.6 Flow diagram summarising the purification of PRAMP cyclohydrolase.

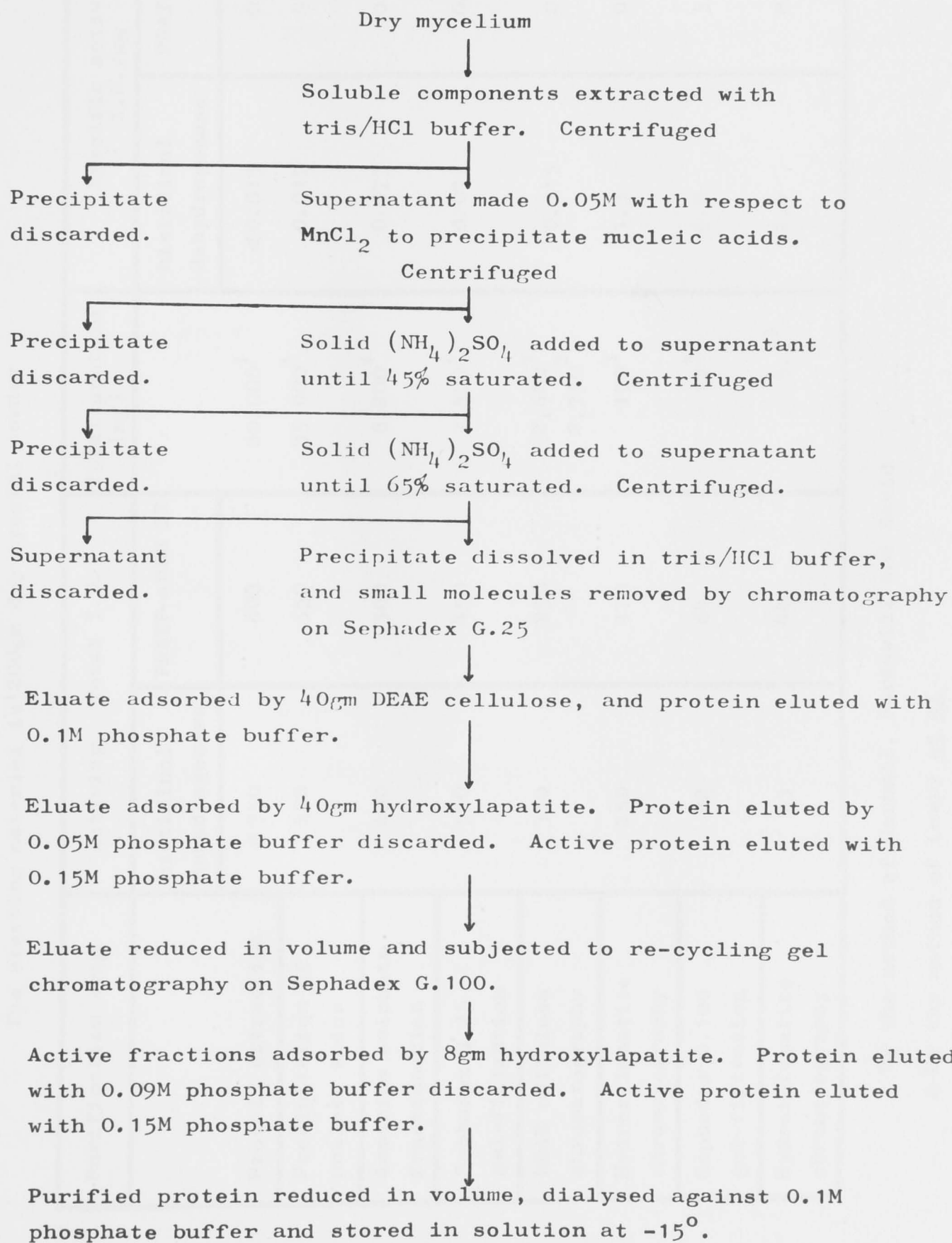


Table 1.6 Purification of PRAMP cyclohydrolase

The starting material is 200gm dry myceial powder

Purification step	Activity, Total I.U.		Total protein (mg)	Specific activity I.U./mg	
	histidinol dehydrogenase	PRATP→BBM II		histidinol dehydrogenase	PRATP→BBM II
Protein extraction	≤ 750	600	60,000 ¹	≤ 0.013	0.01
Precipitation of nucleic acids	600	550	35,000 ¹	0.017	0.015
Ammonium sulphate fractionation	600	500	8,200 ¹	0.073	0.061
Sephadex G.25 gel-filtration	550	450	7,800 ¹	0.071	0.058
DEAE cellulose chromatography	310	230	2,600 ¹ 2,750 ²	0.115	0.085
Hydroxylapatite chromatography	250	170	190 ³	1.3	0.9
Sephadex G.100 gel-filtration	82	64	12 ³	6.8	5.3
Hydroxylapatite chromatography	63	44	5.1 ³	12.4	8.7

1 By the method of Gornall, Bardawill and David

2 By the method of Lowry et al

3 By the method of Lowry et al after dialysis

Steps 1, 2 and 3: Extraction of enzyme, precipitation of nucleic acids and ammonium sulphate fractionation were performed as described by Creaser et al. (1967).¹

Step 4 The ammonium sulphate precipitate obtained from step 3 was dissolved in 150ml 0.05M tris/HCl, pH 7.5, and applied to a 15 x 9cm column of Sephadex G25, previously equilibrated with 0.01M phosphate buffer, pH 6.8. The column was then washed with the same phosphate buffer, and all the eluted protein was collected in a volume of about 300ml. This procedure removes the ammonium sulphate, and prepares the protein for the next step of the purification (removal of the ammonium sulphate by dialysis at this stage results in heavy losses of both activities). This step was performed at room temperature.

Step 5 The protein was mixed with 40 gm of DEAE-cellulose, and the slurry made to 2l. with distilled

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It will be noted that although steps 1 - 3 are identical to those used by Creaser et al. (1967), the yield of protein and histidinol dehydrogenase activity are different (compare tables 1.3 and 1.6). The results reported by Creaser et al. are from their work in Department of Microbiology, Birmingham University, England, where the organism was grown under different conditions from those in Canberra. Growth of Neurospora crassa in Birmingham was carried out at 34°, and in Canberra at 25°.

water. After adsorption of the protein (about 20min), the exchanger was collected by filtration, and washed with 2l. 0.01M phosphate buffer, pH 6.8. This was repeated three times. The exchanger was then made into a slurry with 1 litre of the same phosphate buffer, and packed into a 3cm diameter glass column. The column was eluted with 0.1M phosphate buffer, pH 6.8. Activity was eluted at the buffer front together with a dark protein band. This dark band, and the following 200ml were collected.

Step 6 The protein solution was diluted fourfold with distilled water to give a phosphate molarity of 0.025, and the solution made 0.2% (v/v) with respect to 2-mercaptoethanol. This solution was added to 40gm of hydroxylapatite, and the suspension stirred for 10min. The suspension was then filtered, and the pad of hydroxylapatite washed three times with 300ml 0.05M phosphate buffer, pH 6.8. The hydroxylapatite was packed into a 3cm diameter glass column, and the column eluted with 0.15M phosphate buffer, pH 6.8, containing 0.2% 2-mercaptoethanol. Enzyme activity was eluted at the buffer front, and 100ml of eluate was collected.

the suspension stirred for 10min. The material was

After standing overnight a white precipitate developed in the protein solution. This was removed by centrifuging for 15min at 10,000 x g, and the precipitate was discarded. No losses of activity resulted.

Step 7 The protein solution was reduced to a volume of approximately 8ml by ultrafiltration, and applied to a 100 x 5 cm column of Sephadex G100. The column was equilibrated and eluted with 0.1M phosphate buffer pH 6.8, containing 0.2% 2-mercaptoethanol. The column was maintained at 10° by water jacketing, and the flow rate was 70ml/hr in the upward direction, this flow rate being maintained by a peristaltic pump. Fig. 2.6 shows the elution of protein and enzyme activity. The eluate corresponding to the shaded area in fig. 2.6 was recycled into the bottom of the column, and when this material was eluted for the second time it was collected in 5ml fractions. The fractions between the arrows were pooled.

Step 8 The material obtained from step 7 was diluted with three times its own volume of water, and made to 0.1% with respect to 2-mercaptoethanol. The solution was then added to 8gm of hydroxylapatite, and the suspension stirred for 10min. The material was

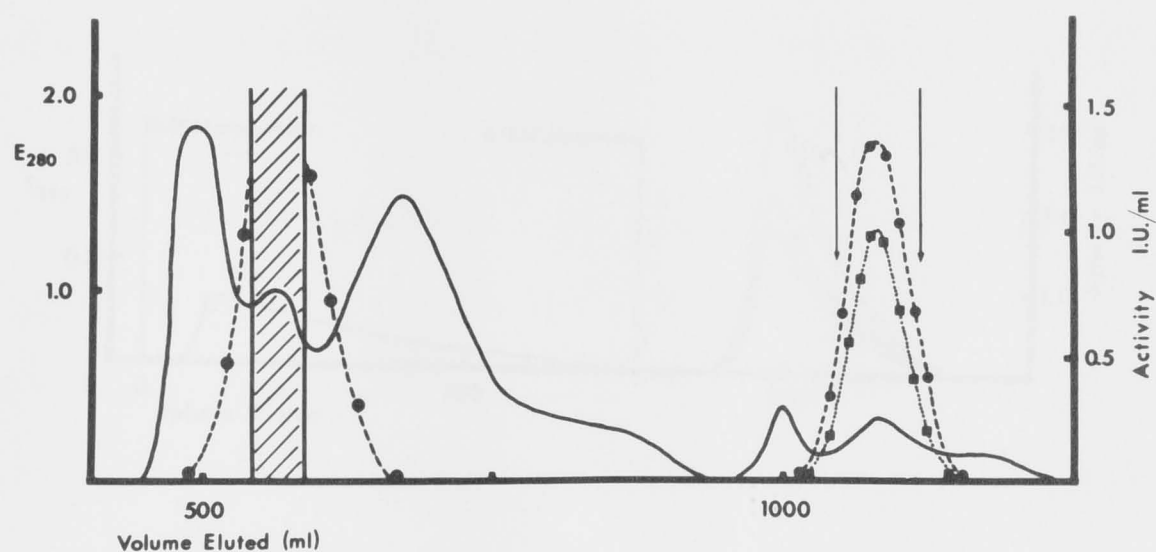


Figure 2.6 Purification step 7: Recycling gel filtration

An 8ml sample was chromatographed on a 1700ml Sephadex G100 column. The protein eluate was monitored (—), and either collected or re-cycled (shaded area). After two cycles the sample was completely eluted and the eluate was assayed for histidinol dehydrogenase activity (•-----•), and for ability to convert PRATP to BBM II (■.....■).

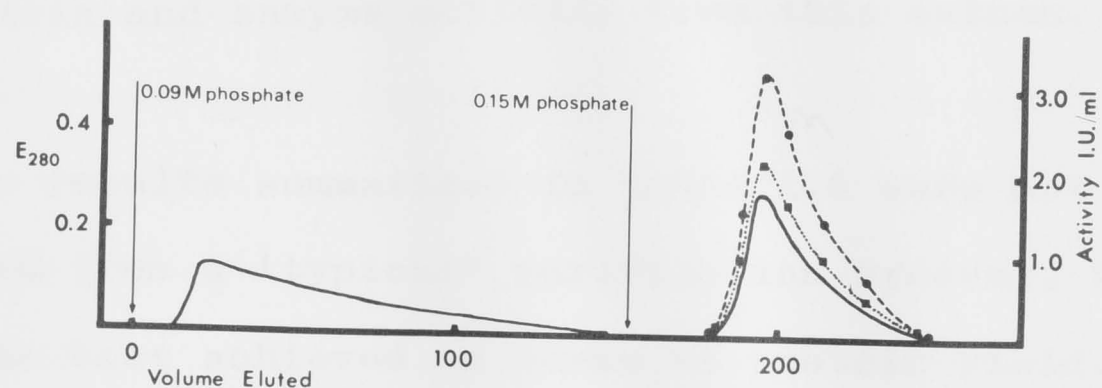


Figure 3.6 Purification step 8.

Hydroxylapatite chromatography.

The sample was mixed with 8gm of hydroxylapatite and the suspension packed into a 1.5cm diameter glass column. The column was then washed with 150ml 0.09M phosphate buffer and with 100ml 0.15M phosphate buffer. The eluate was monitored (—), and the fractions assayed for histidinol dehydrogenase activity (•----•) and for ability to convert PRATP to BBM II (■.....■).

packed into a 1.5cm glass column, and the column was eluted with 150ml 0.09M phosphate buffer, pH 6.8, and then with 100ml 0.15M phosphate buffer, pH 6.8, containing 0.1% 2-mercaptoethanol. All enzyme activity was eluted in about 60ml of the higher molarity buffer. Fig. 3.6 shows the elution pattern of protein and enzyme activity from this column.

Yield

The results summarised in table 1.6 were not obtained from a 'typical' purification process, but from the best achieved in terms of protein yield. Yields of protein from 200gm of dry mycelium varied from 5mg to 2.5mg. The purified protein always contained the two enzymic activities in a ratio of 3:2 (histidinol dehydrogenase : PRATP \rightarrow BBM II), and this is approximately the same ratio as is found in the crude extract. The yield of enzyme activity in the purified preparation was 5-10% of that found in the crude extract.

Comments on the purification procedure

The use of high concentrations of 2-mercaptoethanol, although essential, was in some respects inconvenient. Protein solutions containing the concentrations of 2-mercaptoethanol used during fractionation

procedures, cannot be frozen without considerable precipitation of protein and loss of enzyme activity, and for this reason the purification must be completed without freezing protein solutions containing 2-mercaptoethanol. Unfrozen solutions were very susceptible to attack by microorganisms, so that it was essential to complete the purification as quickly as possible. In practice steps 1, 2 and 3 were performed in one day. The ammonium sulphate precipitate could be stored for one week at -15° without loss of activity. The remaining stages were completed in four days. The purified protein is more stable than at any stage during the purification, since the 2-mercaptoethanol may be removed by dialysis, and the protein solution frozen and thawed several times without loss of activity.

The purification procedure was not entirely reproducible. All preparations were subjected to analytical ultracentrifugation (see 'purity criteria'), and one preparation in three was found to contain a rapidly sedimenting component. Such preparations were discarded. Inability to obtain consistently pure preparations was probably due to the difficulty of precisely repeating purification step 7 (recycling gel filtration). Consideration of fig. 2.6 shows that

a very small volume is recycled into the column (about 40ml) and it was not found possible to recycle the correct fraction with an accuracy of better than ± 5 ml. The need to test all preparations for the presence of impurity, reduced the effective yield of protein obtained from each preparation. In order to obtain purified preparations at a suitably high concentration for analytical ultracentrifugation, protein solutions resulting from the final step of the purification were reduced to a volume of about 1ml by ultrafiltration. Only about 50% of the protein was recovered after this treatment.

(b) Purity criteria

Methods

Ultracentrifugation

Analytical ultracentrifugation was carried out using a Beckmann Model E analytical ultracentrifuge. All experiments were performed using a 12mm double sector centrepiece in order to obtain a Schlieren baseline. The rotor was operated at 50740 rpm.

Sucrose density centrifugation was carried out according to the method of Martin and Ames (1961) using a 4.5ml 5-20% linear sucrose gradient. The gradient was prepared using a two-chamber mixing device, and

was allowed to stand for 4 hr at 4° before use. Samples were applied in a volume of 0.1ml. All experiments were performed in a Beckmann L-2 preparative ultracentrifuge using an S.W.39 rotor operated at 38,000rpm for 18hr. The temperature was maintained at 4° . After centrifuging, each tube was pierced and single drop fractions were collected. Each tube yielded 74-76 drops. Enzyme activities were assayed as described previously except that the buffer was added to the tube containing the fraction. The contents of the tube were then thoroughly mixed and transferred to the assay cuvette. The reaction was initiated as usual by the addition of substrate. For the purposes of protein estimation, 3-drop fractions were collected and made to 1ml with water.

Gel electrophoresis

Starch gel electrophoresis was performed according to the method of Smithies (1955), using a horizontal gel of dimensions 20 x 6 x 0.4cm. Hydrolysed starch was obtained from Connought Laboratories, Toronto, and gels were prepared using starch concentrations 1% higher than those recommended by the manufacturer. Gels were prepared in 0.05M tris/HCl, pH 8.7, and the electrode vessels contained

the same buffer at a concentration of 0.125M. Electrophoresis was carried out for 12hr at 20V/cm. The ambient temperature was 4°.

After each electrophoresis run the gels were sliced and treated in the following way.

- (1) Protein was fixed and stained with a 1% solution of amido black in methanol/acetic acid/water, 50/20/50 (v/v). Excess stain was removed by washing with the same solvent.
- (2) Histidinol dehydrogenase activity was located by the method of Davidson et al. (1965), using histidinol as the proton donor. The assay solution consisted of the following reagents dissolved in 100ml 0.1M tris/HCl, pH 8.7: 10mg phenazine methosulphate; 10mg MTT tetrazolium; 100mg NAD and 100mg histidinol. The assay solution was poured onto the gel, and purple bands of reduced dye appeared after 1-3 min at room temperature. The reaction was stopped by replacing the assay solution with 2% (v/v) acetic acid.
- (3) Cyclohydrolase and pyrophosphohydrolase activities were located by cutting the gel into 4mm strips, and grinding each strip into 1ml 0.05M tris/HCl, pH 8.5, containing 2mM MgCl₂ and 5mM 2-mercaptoethanol. 0.15umoles PRATP was added, and the mixture incubated for 15min

at 37° . The reaction was stopped by the addition of 0.5ml N-HCl, and the starch particles removed by centrifuging for 20min at 15,000 x g. The supernatant was then assayed for BBM II as previously described. Strips of gel ground into a suspension were also tested by the mixed extract method, to ensure that no fraction contained one of the two enzyme activities independent of the other.

The method for locating protein results in shrinkage of the gel, whereas the procedure for locating histidinol dehydrogenase activity does not. Where gels have been photographed, the prints have been differentially enlarged so that gels stained by the two methods appear the same size.

Poly-acrylamide gel electrophoresis was performed using vertical gel electrophoresis apparatus supplied by E-C Apparatus Corporation, Phil., P.A.. 3mm gels were prepared using 5% Cyanogum 41 (E-C Apparatus Corp.), which consists of 95% acrylamide and 5% N,N^{1} -methylene-bis-acrylamide. Gels were polymerised with ammonium persulphate and TMED (N,N,N^{1},N^{1} -tetramethylethylenediamine), each at a concentration of 0.1%. Gels and electrode vessels contained 0.1M tris/HCl, pH 8.7. The gels were maintained at room

temperature by passing tap water through the cooling plates, and the gels were pre-run for one hour, or until the voltage was stable. The sample (0.01ml) was then applied, and the voltage adjusted to give a potential difference of 20V/cm. A serum protein sample, previously stained with bromophenol blue, was run in parallel during each experiment. The albumin component binds the dye, and the progress of the electrophoresis was followed by the migration of the stained albumin band.

After about 4hr the gels were removed, and stained for protein and enzyme activity by the methods described for starch gels.

Results

Analytical ultracentrifugation

Fig. 4.6 shows the sedimentation behaviour of a 0.27% solution of the purified protein in 0.1M phosphate buffer, pH 6.8. At this concentration the protein sediments as a single boundary. The Schlieren pattern is symmetrical except for a slight 'tail' on the solvent side of the boundary.

Sucrose density gradient centrifugation

Initial experiments were performed using gradients prepared in tris/HCl buffers, pH 8.5, according to the

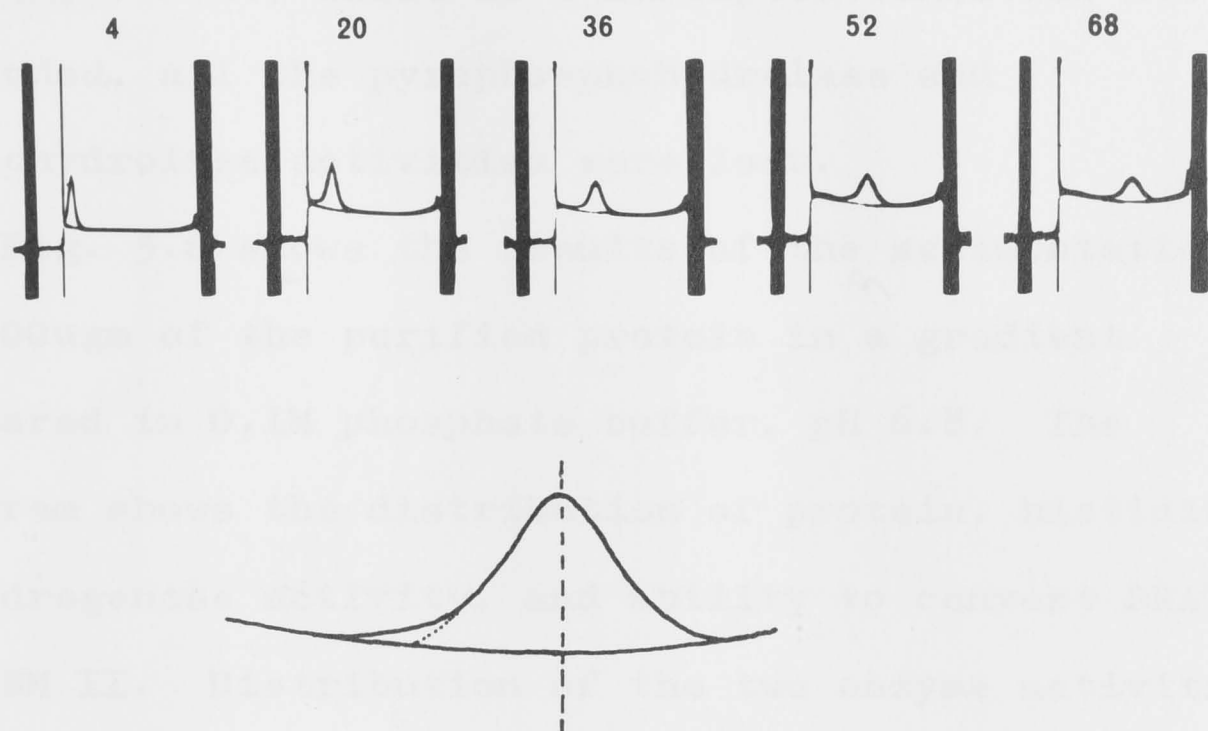


Figure 4.6 Sedimentation of PRAMP cyclohydrolase

A 0.27% solution of the purified protein in 0.1M sodium/potassium phosphate, pH 6.8 was centrifuged at 50740rpm. at 21°. Sedimentation is from left to right. Numbers above each frame give the time in minutes after reaching speed. The lower diagram is a tracing of an enlarged print of the frame taken after 68min. The dotted line represents the mirror image of the right hand half of the Schlieren peak (where the dashed line bisects the peak).

method of Martin and Ames (1961). It was found, however, that if this buffer system was used in the presence of 2-mercaptoethanol, 95% of the histidinol dehydrogenase activity was lost during the course of the experiment, while if 2-mercaptoethanol was not included, all the pyrophosphohydrolase and cyclohydrolase activities were lost.

Fig. 5.6 shows the results of the sedimentation of 200ugm of the purified protein in a gradient prepared in 0.1M phosphate buffer, pH 6.8. The diagram shows the distribution of protein, histidinol dehydrogenase activity, and ability to convert PRATP to BBM II. Distribution of the two enzyme activities were obtained by assaying alternate drops from a gradient which contained 0.1% 2-mercaptoethanol in order to retain enzyme activity. The histogram showing protein distribution was obtained by assaying 3-drop fractions from a gradient, centrifuged in parallel, which contained no 2-mercaptoethanol, because this was found to interfere with the protein assay method. Thus the experimental conditions are not identical in the two tubes, and the results obtained from the two tubes are not strictly comparable. However, the distributions of protein and enzyme activity are so similar it seems unlikely that

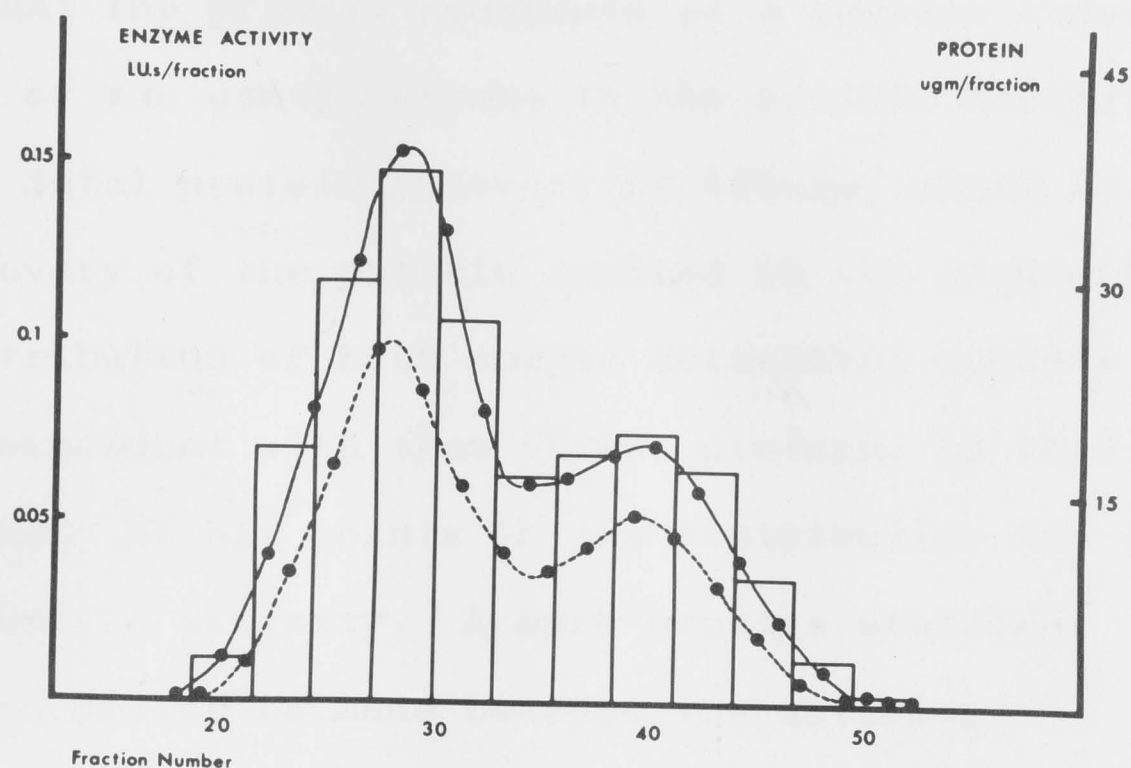


Figure 5.6 Sucrose density gradient centrifugation of PRAMP cyclohydrolase

The distribution is shown of a 200ugm (0.1ml) sample after centrifugation for 18hr at 38,000 rpm. The distributions of histidinol dehydrogenase activity (•—•), and ability to convert PRATP to BBM II (•-----•) were obtained by assaying alternate 1-drop fractions from the same gradient. The protein histogram was obtained by assaying 3-drop fractions from a gradient centrifuged in parallel. Sedimentation is from right to left.

2-mercaptoethanol has any effect on the sedimentation behaviour of the protein.

It is clear that under the conditions of this experiment the protein sediments as a complex zone. The sum of the contributions to the protein histogram gives a total protein recovery of 196ugm, which is a 98% recovery of the protein applied to the gradient. The distribution of both enzyme activities appears to be coincident with that of the protein, so that the protein at all points on the distribution has the same specific activity. A more precise statement than this cannot be made because the activity distributions are obtained from discontinuous 1-drop fractions, while the protein distribution is obtained from continuous 3-drop fractions. Nevertheless, a visual examination alone of fig. 5.6 is sufficient to establish that the three distributions are very nearly coincident.

Starch gel electrophoresis

Fig. 6.6 shows the combined results of two electrophoresis experiments. In one experiment the gel contained 0.1% 2-mercaptoethanol, which was added after de-gassing of the molten gel, and in the other experiment the gel contained no 2-mercaptoethanol. In all other respects the experiments were identical.

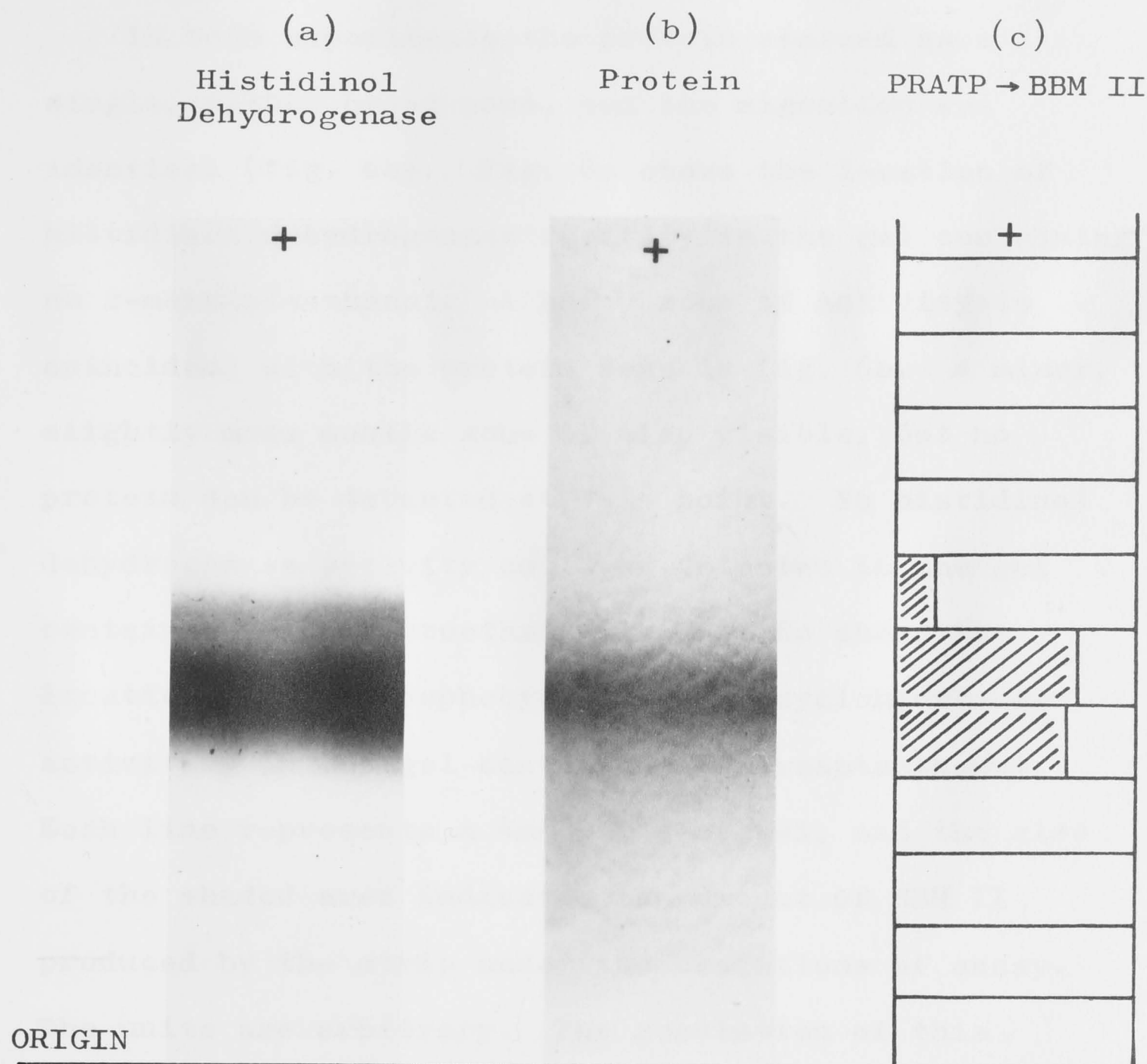


Figure 6.6 Starch gel electrophoresis of
PRAMP cyclohydrolase

Conditions of experiment are described in the text.

- (a) Gel stained for histidinol dehydrogenase activity
- (b) Gel stained for protein
- (c) Gel tested for ability to convert PRATP to BBM II.
The gel was cut into 4mm strips. The width of the shaded area indicates the amount of BBM II produced by the corresponding strip of gel.

In both experiments the protein stained as a single, rather broad zone, and the migration was identical (fig. 6b). Fig. 6a shows the location of histidinol dehydrogenase activity in the gel containing no 2-mercaptoethanol. A heavy zone of activity is coincident with the protein zone in fig. 6b. A minor, slightly more mobile zone is also visible, but no protein can be detected at this point. No histidinol dehydrogenase activity could be detected in the gel containing 2-mercaptoethanol.¹ Fig. 6c shows the location of pyrophosphohydrolase and cyclohydrolase activities in the gel containing 2-mercaptoethanol. Each line represents a 4mm strip of gel, and the size of the shaded area indicates the amount of BBM II produced by the strip under the conditions of assay. The units are arbitrary. The resolution of this method of location was low because large strips of

1

The 2-mercaptoethanol acts as a very weak proton donor in this system, and the entire gel is stained purple after about 20min at room temperature. However, no histidinol dehydrogenase activity was detected during the first ten minutes after the addition of the assay solution. It has been noted already that 2-mercaptoethanol, in the presence of tris/HCl buffers results in heavy losses of histidinol dehydrogenase activity during DEAE and TEAE-cellulose chromatography and sucrose density gradient centrifugation.

gel were required in order to obtain detectable levels of activity. However, it is clear that these activities have approximately the same distribution in this gel as does histidinol dehydrogenase activity in the corresponding gel. No pyrophosphohydrolase or cyclohydrolase activity could be detected in the gel containing no 2-mercaptoethanol.

Acrylamide gel electrophoresis

The results are illustrated in fig. 7.6, and it is clear that they are similar to those obtained by starch gel electrophoresis. Two zones of histidinol dehydrogenase activity are present, and these correspond to two zones of protein. The more mobile of the two protein zones was a minor component, and was only just detectable. The protein zones were unusually broad. A serum protein sample electrophoresed in parallel gave the sharp zones usually obtained by this technique, and the broad zones obtained after starch and acrylamide gel electrophoresis of PRAMP cyclohydrolase are probably, therefore, a property of the protein rather than of the technique used.

Location of histidinol dehydrogenase activity was more difficult on acrylamide than on starch gels.

Appearance of the reduced dye required about 20min at

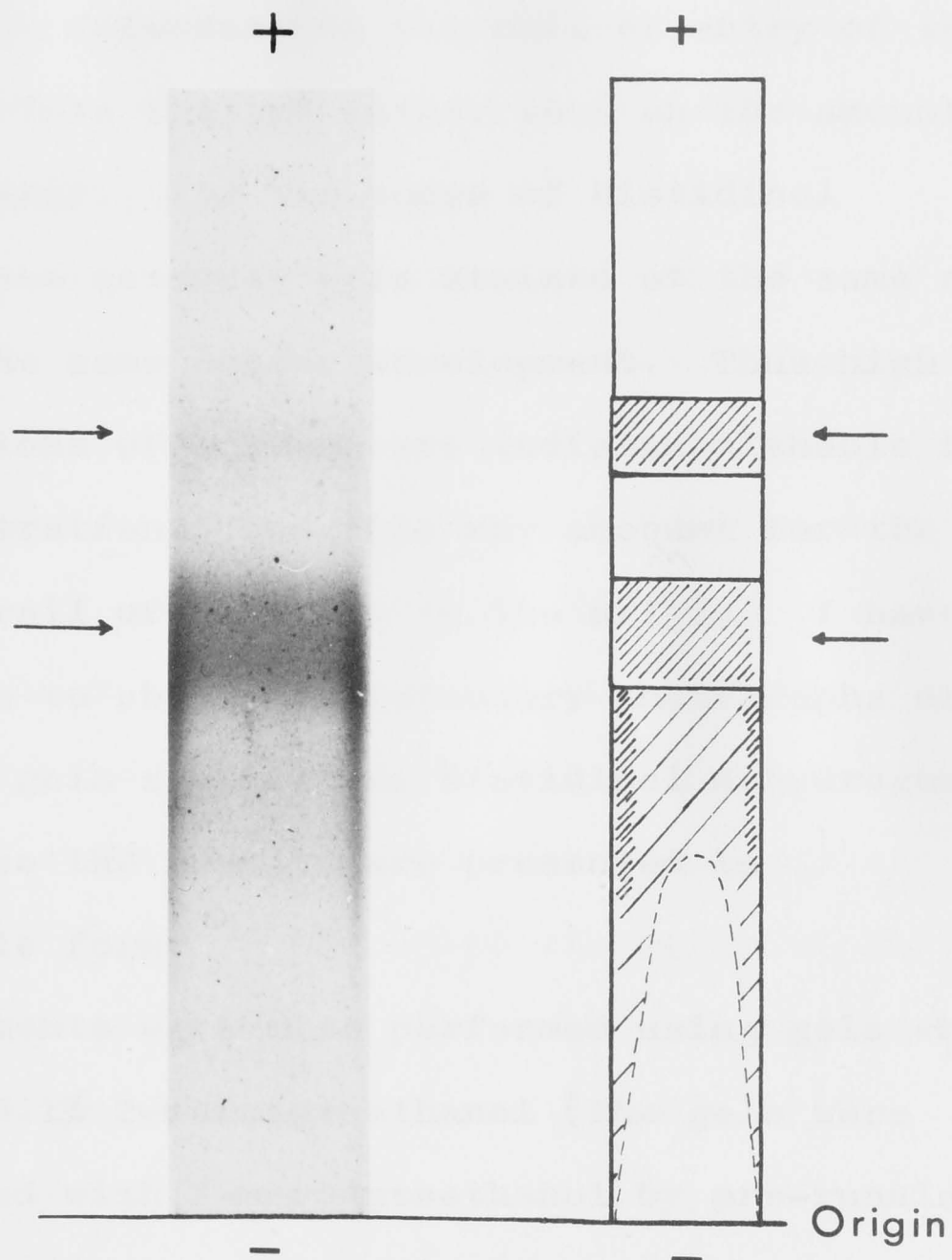


Figure 7.6 Acrylamide gel electrophoresis of
PRAMP cyclohydrolase

30ug (0.01ml) samples were electrophoresed in parallel for 4hr at 20v/cm. The gels were stained for protein (left), and for histidinol dehydrogenase activity (right). The density of shading indicates the density of reduced dye.

room temperature, and the colour development observed seemed to be dependent on the rate of entry of the substrates into the gel rather than on the amount of enzyme present. The two zones of histidinol dehydrogenase activity were stained at the same rate and gave the same colour development. Thus high concentrations of enzyme were indistinguishable from low concentrations, and this may account for the observed trail of activity to the origin. I have been unable to obtain satisfactory photographs of acrylamide gels stained for histidinol dehydrogenase activity, so the results are presented in diagrammatic form.

Experiments were also performed using gels which contained 0.1% 2-mercaptoethanol (the gels were equilibrated with 2-mercaptoethanol by pre-running for 3hr with 0.1% 2-mercaptoethanol present in the electrode vessels). Neither these gels, nor those which contained no 2-mercaptoethanol, contained any detectable pyrophosphohydrolase or cyclohydrolase activity. This result is probably due to the high cross linkage of acrylamide gels, and the consequent low rates of diffusion of enzyme out of the gel (or of substrate in and product out). The presence of

2-mercaptoethanol had no effect on the observed protein distribution. Histidinol dehydrogenase activity could not be detected in gels which contained 2-mercaptoethanol.

Discussion of purity criteria

Ideally, a demonstration of the purity of a preparation of macromolecules is obtained by physical means; by showing that all, or a very large proportion, of the molecules in the preparation behave identically in centrifugal and electrical fields. By these means it can be shown that all the molecules in the preparation have the same molecular weight, diffusion coefficient and overall electrical charge under various conditions of pH, ionic strength and at different concentrations. The sub-units of many polymeric proteins, however, are capable of associating and dissociating so that 'pure' preparations of these proteins are not collections of identical molecules, but equilibrium mixtures of different molecules. A superficial examination of an equilibrium mixture may often result in the conclusion that a single physical species is present. A mixture in rapid equilibrium may sediment as a single boundary in a centrifugal field, and the rate of sedimentation will be the average rate for the

components of the mixture. A characteristic of such mixtures, however, is that this rate will be concentration dependent. The contribution of associated and dissociated sub-units in the equilibrium mixture will depend on the overall concentration, and at low concentrations the contribution of dissociated sub-units will increase. A boundary analysis of such an equilibrium mixture should therefore show that the boundary is not symmetrical, because the molecules on the solvent side of the boundary (where the concentration is low) should be behaving differently from the molecules on the solute side of the boundary (where the concentration is high). Such an analysis demonstrates the heterogeneity of the preparation. The problem then becomes to demonstrate that all the heterogeneity observed is due to an equilibrium mixture, and not to the presence of a non-interacting impurity. Such a demonstration by physical means may be very difficult, and in the case of complex equilibrium mixtures, may prove impossible.

The experiments performed here to establish the purity of PRAMP cyclohydrolase preparations show that the preparations do not contain a single physical species.

The Schlieren pattern obtained during sedimentation in the analytical ultracentrifuge shows a 'tail' on the solvent side of the boundary. The boundary is therefore not perfectly symmetrical. Sucrose density gradient centrifugation results in a complex protein distribution. Acrylamide gel electrophoresis does not result in a single, sharp, protein zone. These results establish that several molecular species are present. The arguments that the PRAMP cyclohydrolase preparation is 'pure' are as follows.

- (1) The protein sediments as a single boundary in the ultracentrifuge. This rules out the possibility of the presence of an impurity with a very different sedimentation coefficient. The assymetry of the boundary could be due to the presence of an impurity with a very similar sedimentation coefficient, or to the concentration dependent interaction of sub-units.
- (2) The protein sediments as a complex zone in sucrose density gradients. Each point on the protein distribution represents the sum of the contributions of the components at that point. The relative contributions of each of the species present must be different at different parts of the distribution. However, the specific activities are identical throughout the protein distribution, so that all the components present must,

potentially, have the same activity per unit weight ('potentially' because the equilibrium at any point in the distribution is doubtless altered by being subjected to assay conditions). It follows from this that the polydispersity observed after density gradient centrifugation is due to the interaction of chemically indential units. This is not to say that only one type of polypeptide exists in the preparation. Each 'unit' might, for instance, consist of a stable dimer composed of two different polypeptides. The view that the observed heterogeneity of PRAMP cyclohydrolase preparations is due to the association and dissociation of subunits is supported by the fact that all the detectable protein after starch gel electrophoresis had pyrophosphohydrolase, cyclohydrolase and dehydrogenase activities.

Much of this argument rests on the finding that different components in the preparation could not be differentiated on the basis of catalytic activity. The weight which may be placed on this kind of evidence depends on the resolution of the fractionation techniques used. A limitation which is placed on the fractionation techniques is that they must not result in heavy losses of enzyme activity, since the object

of the fractionation is to demonstrate the homogeneity of the preparation with respect to activity. This is especially inconvenient in the case of electrophoresis. Electrophoresis of PRAMP cyclohydrolase at pHs above 9 and below 6 results in the loss of all enzyme activity, while at pHs close to neutrality the protein migrates so slowly that the resolution of the technique is very low. In practice, therefore, satisfactory results can only be obtained in the pH range 8 - 9. Furthermore the range of buffer systems which can be used is limited. Buffer systems commonly used for gel electrophoresis experiments are tris/EDTA/boric acid, boric acid/LiOH and boric acid/EDTA/NaOH (see for instance Bodman, 1960). These buffer systems usually give separations of higher resolution than tris/HCl buffers (Leggett Bailey, 1967). However, all of the former buffer systems were found to destroy histidinol dehydrogenase, pyrophosphohydrolase and cyclohydrolase activities during starch gel electrophoresis.

No criterion or combination of criteria can demonstrate the absolute purity of a preparation of macromolecules. However, many of the techniques available for demonstrating physical homogeneity are of sufficiently high resolution that the possibility

of high levels of impurity (>5%) remaining undetected is very small. The methods used to demonstrate the purity of PRAMP cyclohydrolase preparations are, perforce, of lower resolution, but a number of methods have been used, and there is a strong case for considering the protein to be pure.

The action of 2-mercaptoethanol

It is clear that 2-mercaptoethanol plays a central role in maintaining PRAMP cyclohydrolase in a structural form which allows it to catalyse the cyclohydrolase and pyrophosphohydrolase reactions. The inclusion of 2-mercaptoethanol was essential during many of the purification steps. Similarly it was found to be essential to include 2-mercaptoethanol in several of the experiments described under 'purity criteria', because sucrose density gradient centrifugation and starch gel electrophoresis resulted in the total loss of pyrophosphohydrolase and cyclohydrolase activities if 2-mercaptoethanol was not included. It was also noted that if a fractionation procedure was carried out in tris/HCl buffers, pH 8.0 - 8.5, the presence of 2-mercaptoethanol resulted in heavy losses of histidinol dehydrogenase activity. This

was true in the case of TEAE-cellulose chromatography, starch gel electrophoresis and sucrose density gradient centrifugation. No losses of histidinol dehydrogenase activity occurred when these experiments were performed in phosphate buffers, pH 6.8. However, the purified protein may be subjected to dialysis against phosphate buffers, pH 6.8, or tris/HCl buffers, pH 8.5, both in the presence or absence of 2-mercaptoethanol without any detectable loss of histidinol dehydrogenase, pyrophosphohydrolase or cyclohydrolase activities. Therefore losses of activity are not solely due to the interaction of protein, 2-mercaptoethanol and buffer. In the case of ion exchange chromatography, it can be argued that losses of activity result from some interaction involving the charged groups on the exchanger. No such explanation can be used in the case of starch gel electrophoresis and sucrose density centrifugation, because the starch and the sucrose should act as inert supports in these fractionation techniques. It seems, therefore, that the process of fractionation itself is responsible for inactivation of the protein, and it follows that losses of activity result from the separation of different components in the

preparation. However, this seems unlikely because 2-mercaptoethanol has no detectable effect on the protein distribution which results from starch gel or acrylamide gel electrophoresis of PRAMP cyclohydrolase. Thus if the presence of 2-mercaptoethanol does result in the separation of different components, then one of the components must be sufficiently small as to be undetectable, and must have so small a charge effect that its removal from the major component has no effect on the migration rate of the latter.

The action of 2-mercaptoethanol in partially inducing pyrophosphohydrolase and cyclohydrolase activities in histidinol dehydrogenase preparations is also unknown. It is unlikely that this action is merely to reduce disulphide linkages because dithiothreitol could not replace 2-mercaptoethanol in the reactivation procedure. Dithiothreitol reduces disulphide linkages more efficiently than 2-mercaptoethanol because the oxidation of dithiothreitol results in the formation of a stable cyclic compound which has no oxidising potential (Cleland, 1964). It is probable that the reversible nature of the action of 2-mercaptoethanol is essential to the mechanism of reactivation. Thus the protein may be allowed a measure of conformational

flexibility in the presence of 2-mercaptoethanol; a flexibility which it does not have under non-reducing conditions, or under strongly reducing conditions such as those imposed by dithiothreitol.

PRAMP cyclohydrolase

Introduction

It is apparent that a protein carrying histidinol dehydrogenase activity may be purified from *Neurospora crassa* by two methods. One method, that of Creaser *et al.* (1967), results in a protein which catalyzes only the oxidation of histidinol to histidine. The other method, described in Chapter 6, results in a protein which catalyzes three quite different reactions: the de-phosphorylation of PRATP to give PRAMP; the hydrolysis of PRAMP to BBM II; and the oxidation of histidinol to histidine. The tri-functional form has been named PRAMP cyclohydrolase. There are two ways in which this result might be explained. Either (1) histidinol dehydrogenase and PRAMP cyclohydrolase are chemically identical, and the disparity in catalytic properties is due to differences in the physical structure of the two molecules, or (2) PRAMP cyclohydrolase and histidinol dehydrogenase have a different chemical composition.

CHAPTER 7

A comparison of some physical and chemical
properties of histidinol dehydrogenase and
PRAMP cyclohydrolase

Introduction

It is apparent that a protein carrying histidinol dehydrogenase activity may be purified from Neurospora crassa by two methods. One method, that of Creaser et al. (1967), results in a protein which catalyses only the oxidation of histidinol to histidine. The other method, described in Chapter 6, results in a protein which catalyses three quite different reactions: the de-phosphorylation of PRATP to give PRAMP; the hydrolysis of PRAMP to BBM II, and the oxidation of histidinol to histidine. The tri-functional form has been named PRAMP cyclohydrolase. There are two ways in which this result might be explained. Either (1) histidinol dehydrogenase and PRAMP cyclohydrolase are chemically identical, and the disparity in catalytic properties is due to differences in the physical structure of the two molecules, or (2) PRAMP cyclohydrolase and histidinol dehydrogenase have a different chemical composition.

It is clear that since PRAMP cyclohydrolase has all three catalytic activities, it must contain all the chemical 'information' that histidinol dehydrogenase contains, and any differences in chemical properties must be due to the presence of an additional component in PRAMP cyclohydrolase.

There is considerable indirect evidence that these two proteins are chemically identical.

(1) PRAMP cyclohydrolase must be specified by the entire his-3 locus (with the possible exception of an operator region) since the protein carries the three catalytic functions controlled by this locus. Bennett and Creaser (1967) showed that histidinol dehydrogenase from a his-3 mutant which lacked pyrophosphohydrolase or cyclohydrolase activities, contained a single amino acid replacement when compared with the wild type enzyme. This observation provides evidence that the purified histidinol dehydrogenase is also specified by the entire his-3 locus, and it is clear that if PRAMP cyclohydrolase and histidinol dehydrogenase are specified by the same genetic material, then the two proteins must have the same amino acid sequence. Similar conclusions were drawn from an investigation of the kinetic properties of histidinol dehydrogenase from

a wide range of his-3 mutants (Creaser, Bennett and Drysdale, 1965). However, as was argued in Chapter 5, since the kinetic analyses were performed using partially purified enzymes, the conclusions are not necessarily valid for the purified histidinol dehydrogenase.

(2) Histidinol dehydrogenase preparations can be induced to catalyse the pyrophosphohydrolase and cyclohydrolase reactions by treatment with 2-mercaptoethanol. The efficiency of this reactivation is very low, but the simplest explanation of the phenomenon is that histidinol dehydrogenase molecules are chemically identical to PRAMP cyclohydrolase molecules, and that treatment with 2-mercaptoethanol renders a small number of them physically identical also.

(3) If the multiple catalytic activities of PRAMP cyclohydrolase are due to the presence of an additional component, then this component must be removed during the purification of histidinol dehydrogenase. However, at no point during the purification of histidinol dehydrogenase does the re-mixing of fractions improve the yield of pyrophosphohydrolase and cyclohydrolase activities.

(4) If PRAMP cyclohydrolase contains an additional component, then the presence of this component has not become apparent from investigations of the behaviour of PRAMP cyclohydrolase in centrifugal and electrical fields. Solutions of the protein at low concentrations show considerable polydispersity, but this polydispersity seems to be due to the interaction of identical units, rather than to the interaction of different chemical components.

Points (3) and (4) provide negative evidence that PRAMP cyclohydrolase does not contain an additional component, and hence that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical.

This evidence is not conclusive and a direct demonstration of chemical identity is clearly required.

Amino acid composition of PRAMP cyclohydrolase and histidinol dehydrogenase

Methods

(a) Preparation of protein for treatment

The purified protein was dialysed twice for 24hr against 2l. of distilled water, and finally for 24hr against 2l of de-ionised water containing about 2gm of Amberlite Monobed Resin (British Drug Houses Ltd.).

The protein solution was then lyophilised and dried in vacuo over concentrated sulphuric acid.

(b) Performic oxidation

Performic acid oxidation (Hirs, 1956; Moore, 1963) was carried out as follows. Performic acid was prepared by the addition of 1ml 30% hydrogen peroxide to 9ml re-distilled formic acid, and the solution was allowed to stand for 1hr at room temperature in a closed cylinder. The solution was then cooled to 0°, and 1ml was added to about 5mg of dry protein. Protein and performic acid were allowed to react for 3hr at 0°. The solution was then diluted to 50ml with de-ionised water, and was lyophilised. The lyophilised protein was dissolved in 1ml formic acid, made to 10ml with de-ionised water, and then lyophilised again to remove all traces of performic acid.

of Spackman, Stein and Moore (1958), and Moore, Spackman

A standard amino acid mixture was subjected to this performic oxidation procedure to find the yields of cysteic acid and methionine sulphone, and to determine any losses of tyrosine and histidine. Yields of all amino acids after performic oxidation are shown in table 1.7. Tryptophan is always destroyed by performic oxidation.

(c) Acid hydrolysis

A performically oxidised preparation of known weight of protein was made to a solution of known volume with a small amount of formic acid at 0°. Aliquots of the solution were dispensed into Pyrex tubes so that each tube contained sufficient protein for two amino acid analyses after hydrolysis (about 0.4mg). The formic acid was then removed under a stream of nitrogen, and 1ml of distilled HCl was added to each tube. The solutions were frozen with liquid nitrogen, thawed out under a stream of nitrogen and immediately sealed. The phials were then incubated at 110°. After 24, 48 or 72hr the phials were removed and opened, and the HCl removed under a stream of nitrogen over a boiling water bath.

(d) Amino acid analysis

Amino acid analysis was performed by the method of Spackman, Stein and Moore (1958), and Moore, Spackman

Table 1.7 Mean values obtained from seven standard analyses of a sample containing 5×10^{-8} moles of each amino acid.
Yields after performic oxidation.

Amino Acid	Mean constant	Standard deviation	Oxidised standard	Percentage yield
Lys	93.81	2.08	94.82	-
His	85.84	1.82	84.40	-
Arg	86.35	1.76	85.81	-
Cysteic acid	89.29	1.47	76.00	84.5%
Asp	83.93	0.67	82.84	-
Thr	88.04	0.52	88.92	-
Ser	94.31	0.58	95.32	-
Glu	89.37	0.96	90.34	-
Pro	21.11	0.54	20.70	-
Gly	86.80	0.74	87.88	-
Ala	89.23	0.89	90.82	-
CyS	44.47	0.84		
Val	88.66	0.98	88.87	-
Met	87.99	0.51		
Ile	85.46	0.79	85.80	-
Leu	91.34	0.86	90.88	-
Tyr	93.79	1.25	83.52	89.0%
Phe	97.07	1.61	96.20	-
Met. sulphone	93.11	1.40	81.00	87.0%

Where no entry is made the oxidised standard gave a value within two standard deviations of the mean constant, and the yield is therefore assumed to be 100%.

and Stein (1958) using a Beckman 120B amino acid analyser. The instrument was fitted with a Honeywell manufactured 'scale expander', which amplified the signal approximately fourfold, and gave linear readings of extinction. Constants and standard deviations for each amino acid were found by analysing standard samples containing 5×10^{-8} moles of each amino acid, plus methionine sulphone and cysteic acid at the same concentration. Mean values and standard deviations for each amino acid calculated from seven standard runs are given in table 1.7.

(e) Estimation of tryptophan

Tryptophan was estimated by the method of Spies and Chambers (1949). The conditions used were as follows. To 0.1ml of protein solution containing 0.2 - 0.3mg protein was added 0.9ml 21.4N- H_2SO_4 containing 3mg ρ -dimethylaminobenzaldehyde. The solution was thoroughly mixed, and incubated at 25° for 15hr. 0.01ml 0.045% sodium nitrite was then added, and the solution incubated for a further 30min at 25° . 3×10^{-8} moles tryptophan gave an E_{590} reading of 0.305.

Results

Amino acid analysis of PRAMP cyclohydrolase

Results of analyses of a performically oxidised preparation of PRAMP cyclohydrolase after hydrolysis

for 24, 48 and 72hr are given in table 2.7. Each value is the mean of a duplicate. Those residues whose yields vary significantly with time of hydrolysis are shown. Yields of serine and threonine decrease with time of hydrolysis, while yields of glutamic acid, valine, leucine and isoleucine increase with time of hydrolysis. Column 5 of table 2.7 gives the corrected estimates of all residues. Estimates of threonine and serine have been obtained by extrapolating to zero time of hydrolysis (see fig. 1.7) and estimates of glutamic acid, valine, leucine and isoleucine are given as equal to or slightly greater than the 72hr yield. Other residues are estimated as the mean value for the three times of hydrolysis, and values for tyrosine, methionine and cysteine have been corrected by the factors given in table 1.7. An estimate of tryptophan is included in the corrected analysis. All the values given are for 75ugm of protein estimated by dry weight prior to performic oxidation. The weight contribution of each residue is given in column 6 of table 2.7, and the sum of the estimated weights of the residues is 72.99ugm, which is a recovery of 97%.

Table 2.7 Amino acid analysis of PRAMP cyclohydrolase

Amino Acid	Residues recovered from 75ugm protein (Moles x 10 ⁻⁸)			95% confidence limits	Corrected values	Weight contribution (ugm)
	24hr	48hr	72hr			
Lys	4.77	4.68	4.65	± 0.21	4.70	6.14
His	1.52	1.47	1.60	± 0.22	1.53	2.30
Arg	2.38	2.35	2.28	± 0.20	2.34	3.70
Cys ¹	0.83	0.80	0.74	± 0.16	0.93 ⁴	0.98
Asp	5.60	5.53	5.64	± 0.09	5.59	6.54
Thr ³	3.78	3.67	3.62	± 0.06	3.90 ⁵	3.98
Ser ³	3.92	3.67	3.36	± 0.07	4.10 ⁵	3.70
Glu ³	7.24	7.36	7.53	± 0.11	≥ 7.53	9.86
Pro	3.49	3.52	3.73	± 0.25	3.58	3.54
Gly	4.67	4.65	4.73	± 0.08	4.68	2.76
Ala	7.29	7.24	7.30	± 0.10	7.28	5.26
Val ³	5.09	5.32	5.45	± 0.11	≥ 5.45	5.50
Met ²	1.24	1.37	1.37	± 0.14	1.53 ⁴	2.03
Ile ³	3.51	3.72	3.82	± 0.09	≥ 3.82	4.39
Leu ³	5.60	5.77	5.86	± 0.10	≥ 5.86	6.74
Tyr	0.51	0.50	0.51	± 0.13	0.57 ⁴	0.94
Phe	2.40	2.52	2.46	± 0.15	2.46	3.67
Trp	--	--	--		0.51 ⁶	0.96
						Total 72.99

1 Estimated as cysteic acid

2 Estimated as methionine sulphone

3 Recoveries vary significantly with time of hydrolysis

4 Corrected for losses due to performic oxidation (see table 1.7)

5 Extrapolated to zero time of hydrolysis (see fig. 1.7)

6 Estimated by the method of Spies and Chambers (1949)

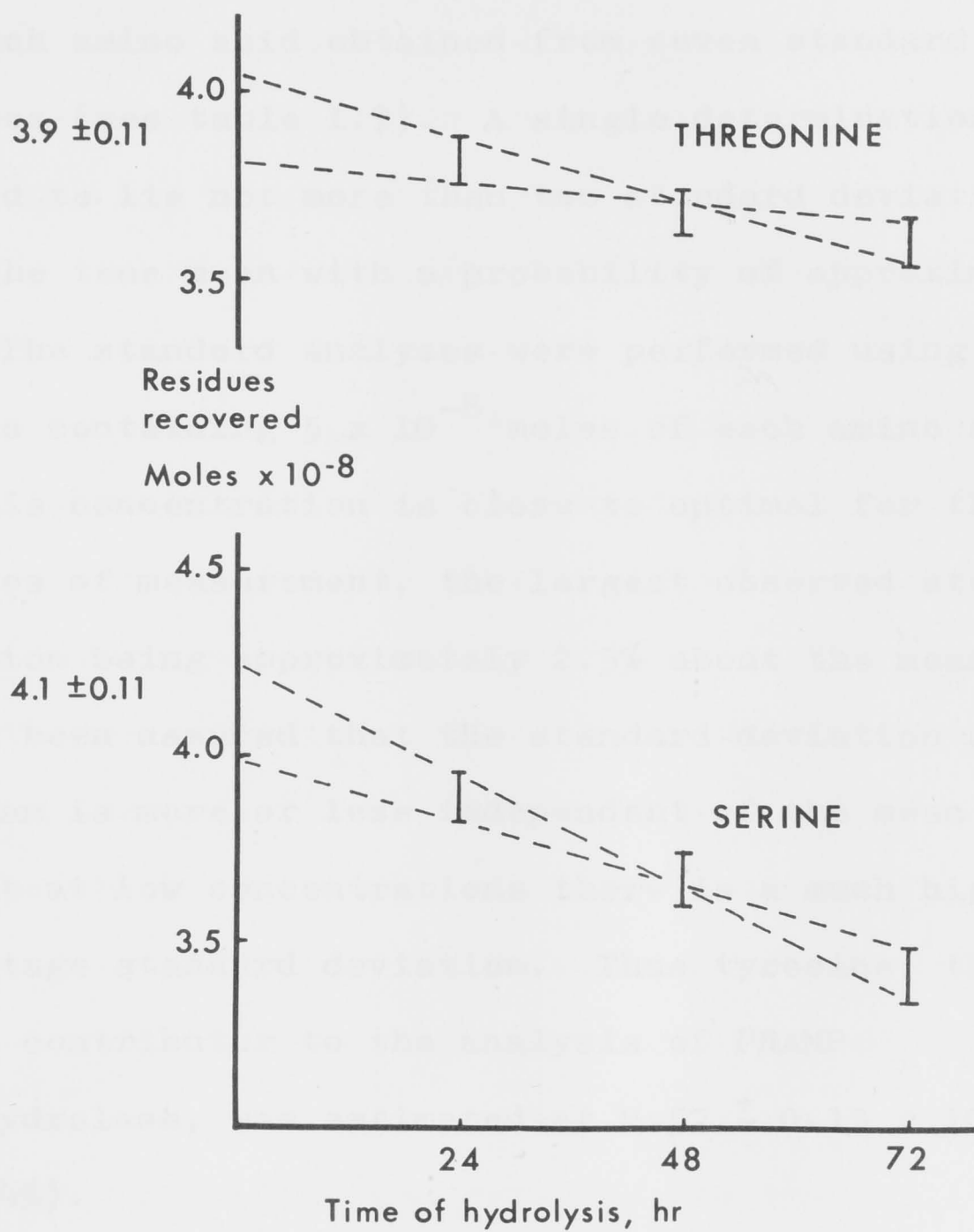


Figure 1.7 Yields of serine and threonine extrapolated to zero time of hydrolysis.

Estimation of 95% confidence limits.

The 95% confidence limits given derive from the observed standard deviation about the mean constant for each amino acid obtained from seven standard analyses (see table 1.7). A single determination is assumed to lie not more than two standard deviations from the true mean with a probability of approximately 95%. The standard analyses were performed using samples containing 5×10^{-8} moles of each amino acid, and this concentration is close to optimal for the purposes of measurement, the largest observed standard deviation being approximately 2.5% about the mean. It has been assumed that the standard deviation about the mean is more or less independent of the mean value, so that at low concentrations there is a much higher percentage standard deviation. Thus tyrosine, the lowest contributor to the analysis of PRAMP cyclohydrolase, was estimated at $0.57 \pm 0.13 \times 10^{-8}$ (or $\pm 24\%$).

Integral contributions of amino acid residues

The minimum chemical molecular weight of a protein can sometimes be calculated by converting the observed ratio of the contributing residues to a ratio in which the contributions of all residues are expressed as integers. In effect this means finding

a multiplication factor which gives the best fit of estimated contributions of each residue to minimum integral contributions. The minimum chemical molecular weight is then the sum of the weights of the residues, or some simple multiple of this sum. Considerable confidence may be placed in such estimations if all residues can be given integral values which lie within the 95% confidence limits of the estimated values (provided, of course, that the multiplication factor is not so large that the 95% confidence limits for most residues are equal to or greater than ± 0.5).

Finding a minimum multiplication factor which converts the estimated values of all residues to integers is a complex task. In practice the values for those residues which contribute at a low level are first converted to integers, and values for all other residues are then multiplied by the same factor to see if they also are converted to integers. Tryptophan, tyrosine, methionine, histidine and cysteine are the smallest contributors to the amino acid composition of PRAMP cyclohydrolase, and it is apparent that the estimated values of these residues may be converted to integers by multiplying by a factor of two. Table 3.7 shows the result of multiplying the estimated values of all

Table 3.7 Estimation of integral composition of PRAMP cyclohydrolase

Amino Acid	Corrected values ¹	Corrected values x 2	Nearest integer values	Weight contribution
Lys	4.70 ± 0.21	9.40 ± 0.42	9	1175
His	1.53 ± 0.22	3.06 ± 0.44	3	471
Arg	2.34 ± 0.20	4.68 ± 0.40	5	790
CyS	0.93 ± 0.16	1.86 ± 0.32	2	210
Asp	5.59 ± 0.09	11.18 ± 0.18	11	1287
Thr	3.90 ± 0.11	7.80 ± 0.22	8	824
Ser	4.10 ± 0.11	8.20 ± 0.22	8	712
Glu	➤ 7.53 ± 0.11	➤ 15.06 ± 0.22	15	1967
Pro	3.58 ± 0.25	7.16 ± 0.50	7	693
Gly	4.68 ± 0.08	9.36 ± 0.16	9 ²	531
Ala	7.28 ± 0.10	14.56 ± 0.20	14-15 ²	949 - 1022
Val	➤ 5.45 ± 0.11	➤ 10.90 ± 0.22	11	1111
Met	1.53 ± 0.14	3.06 ± 0.28	3	399
Ile	➤ 3.82 ± 0.09	➤ 7.64 ± 0.18	8 ³	920
Leu	➤ 5.86 ± 0.10	➤ 11.72 ± 0.20	12 ³	1380
Tyr	0.57 ± 0.13	1.14 ± 0.26	1	165
Phe	2.46 ± 0.15	4.92 ± 0.30	5	745
Trp	0.51	1.02	1	188
			Totals 132 - 133	approx. 14,500

1 From table 2.7

2 Integral value lies outside 95% confidence limits of estimated value

3 Value increased to next highest integer, since yield increases with time of hydrolysis.

residues by a factor of two. This treatment converts the values of all residues except glycine and alanine to molar contributions which lie within two standard deviations of integral values. Leucine and isoleucine fall short of integral values, but since these residues were shown to give increasing yields with increasing time of hydrolysis, it is reasonable to increase these values to the next highest integer. Column 3 of table 3.7 gives the best fitting integral contribution of each residue, and column 4 gives the weight contribution of each residue based on integral values. The total number of residues is 132 - 133, and the total weight is approximately 14,500. The fit of the data to integral values is sufficiently good to predict that the minimum chemical unit has a molecular weight of about 14,500, or some simple multiple of this figure. Comparison of the amino acid composition of PRAMP cyclohydrolase and histidinol dehydrogenase.

Table 4.7 gives a comparison of amino acid analyses of 24hr hydrolysates of PRAMP cyclohydrolase and histidinol dehydrogenase. Both proteins were performically oxidised prior to hydrolysis. The estimated values for all residues in the analysis of histidinol dehydrogenase have been multiplied by a factor which

Table 4.7 Comparison of the amino acid composition of PRAMP cyclohydrolase and histidinol dehydrogenase

Amino Acid	PRAMP cyclohydrolase 24hr hydrolysate ¹	Histidinol dehydrogenase 24hr hydrolysate	Integral Composition	
			PRAMP cyclohydrolase ²	Histidinol dehydrogenase
Lys	4.77	4.60	9	9
His	1.52	1.83	3	4
Arg	2.38	1.94	5	4
CyS	0.83	0.55	2	1 ³
Asp	5.60	6.37	11	13
Thr	3.78	3.78	8	8
Ser	3.92	3.94	8	8
Glu	7.24	6.69	15	14 ⁴
Pro	3.49	2.75	7	5 - 6
Gly	4.67	5.30	9	10 - 11
Ala	7.29	6.68	14 - 15	13 - 14
Val	5.09	4.64	11	10 ⁴
Met	1.24	1.32	3	3 ³
Ile	3.51	3.29	8	7 ⁴
Leu	5.60	5.37	12	12 ⁴
Tyr	0.51	0.62	1	1 ³
Phe	2.40	3.03	5	6
Totals			131 - 132	128 - 131

1 Taken from table 2.7

2 Taken from table 3.7

3 Corrected for losses due to performic oxidation

4 Estimated values taken to the next highest integer on the assumption that the recovery of these residues increases with increasing time of hydrolysis, as is the case for PRAMP cyclohydrolase

gives a value for threonine equal to that estimated for PRAMP cyclohydrolase. It is clear that amino acid analyses of the two proteins give very similar results. A comparison is more easily made of the integral contributions of each residue in the two proteins, and this comparison is made in columns 3 and 4 of table 4.7. Integral contributions of residues in histidinol dehydrogenase are given as the best fitting integers after threonine is given a value of 8 (the same value as previously estimated for PRAMP cyclohydrolase). The integral contributions of all the residues in the two proteins are very similar. The total number of residues in histidinol dehydrogenase is 128 - 131, and in PRAMP cyclohydrolase 131 - 132 (the tryptophan contributions have not been included in this comparison because no estimation of the tryptophan content of histidinol dehydrogenase was made). Only in the case of aspartic acid does the contribution of an amino acid residue differ by more than one unit in the two proteins.

It is reasonable to conclude that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical. The small differences in the amino acid analyses given in table 4.7 could easily be due to the

presence of minor impurities which are known to exist in preparations of histidinol dehydrogenase,¹ and which may be present in PRAMP cyclohydrolase preparations.

(1) Molecular weight estimation.

Estimations of the molecular weight of PRAMP cyclohydrolase and histidinol dehydrogenase were made by the approach to equilibrium method of Schachman (1947), using methods similar to those described by Schachman (1957). All experiments were performed with a Beckman Model E analytical ultracentrifuge using a double sector cup rotor. All measurements were made with Schlieren optical systems obtained at bar angle of 90°. Silica oil was used as an inert, transparent support for the solution and solvent columns.

Approach to equilibrium experiments were performed at rotor speeds of 5500 or 5000 rpm depending on the concentration of the protein sample, and after 10 - 15 min photographs were taken every 1 min for 2 - 3 hr. At the end of the approach to equilibrium experiment,

A review of the approach to equilibrium method is given by Schachman (1959a).

¹

See Appendix II.

Physical properties of PRAMP cyclohydrolase and
histidinol dehydrogenase

Methods

(1) Molecular weight estimations.

Estimations of the molecular weight of PRAMP cyclohydrolase and histidinol dehydrogenase were made by the approach to equilibrium method of Archibald (1947), using methods similar to those described by Schachman (1957).¹ All experiments were performed with a Beckman Model E analytical ultracentrifuge, using a 12mm double sector centrepiece. All measurements were made from Schlieren optical patterns obtained at bar angle of 70° . Silicone oil was used as an inert, transparent support for the solution and solvent columns.

Approach to equilibrium experiments were performed at rotor speeds of 9945 or 13140rpm (depending on the concentration of the protein sample), and after 10 - 15 min photographs were taken every 16min for 2 - 3hr. At the end of the approach to equilibrium experiment,

1

A review of the approach to equilibrium method is given by Schachman (1959a).

the speed was increased to 50740rpm, and further photographs were taken after a complete boundary, with a plateau region on both sides, had been formed. C^0 , the concentration of the protein (in terms of Schlieren optical patterns) at zero time, was then found by measuring the area under the Schlieren peak, and correcting for radial dilution. Determination of C^0 by this method, rather than in a separate centrifuge run in the synthetic boundary cell, has the advantage of increasing the recovery of protein at the end of the experiment (see for example, Nichol and Roy, 1965).

Measurements of the Schlieren patterns were made by transferring enlarged prints to graph paper, and estimations of the molecular weight were made at the meniscus and at the solution/oil interface. Similar values were obtained at these two points in all experiments; the value obtained at the bottom of the cell was never consistently higher than that obtained at the meniscus. However, measurements at the solution/oil interface were found to be less reproducible than those at the meniscus because of the difficulty of precisely locating the intersection of the Schlieren pattern with the interface. For this reason values obtained at the meniscus only are given.

The partial specific volume of PRAMP cyclohydrolase was calculated from the amino acid composition by the method of Cohn and Edsall (1948). The calculation is shown in table 5.7. The value obtained from this calculation is not precisely accurate because glutamine and asparagine residues are estimated as the corresponding acids. However, the amides have the same partial specific volume as the acids, and their molecular weights are only slightly different, so that the error introduced into the calculation is very small. On the assumption that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical, molecular weight estimations have been made using the same partial specific volume for both proteins. Even if this is not the case, the amino acid compositions of the two proteins are so similar that they must have very similar partial specific volumes.

(2) Sedimentation coefficients

Sedimentation coefficients were estimated by the method of Schachman (1957) using a Beckman Model E ultracentrifuge. Protein solutions of concentration greater than 0.15% were centrifuged in a 12mm single sector centrepiece at 52640rpm. Those of concentration less than 0.15% were centrifuged in a 30mm single

Table 5.7 Calculation of the partial specific volume of PRAMP cyclohydrolase

Amino Acid	Integral composition	weight contribution	% weight contribution wi	partial specific volume vi	wi.vi
Lys	9	1175	8.09	0.82	6.634
His	3	471	3.24	0.67	2.171
Arg	5	790	5.44	0.70	3.808
CyS	2	210	1.45	0.63	0.914
Asp	11	1287	8.87	0.59	5.233
Thr	8	824	5.68	0.70	3.976
Ser	8	712	4.91	0.63	3.093
Glu	15	1965	13.54	0.66	8.936
Pro	7	693	4.77	0.76	3.625
Gly	9	531	3.66	0.64	2.342
Ala	14	949	6.54	0.74	4.840
Val	11	1111	7.65	0.86	6.579
Met	3	399	2.75	0.75	2.063
Ile	8	920	6.34	0.90	5.706
Leu	12	1380	9.51	0.90	8.559
Tyr	1	165	1.14	0.71	0.809
Phe	5	745	5.13	0.77	3.950
Trp	1	188	1.30	0.74	0.962
Totals	132	14,515	100.01		74.2

$$\text{Partial specific volume} = 74.2/100.02 = 0.742\text{cc/gm}$$

sector centrepiece at 44770rpm. The temperature at which the rotor was regulated was in all cases between 20 and 21^o. Sedimentation coefficients are expressed as values in water at 20^o ($S_{20,w}$), the observed values having been corrected for the density of the solvent. Temperature corrections for the viscosity of the solvent were made by assuming that the viscosity of the solvent varies with temperature in the same way as does the viscosity of water.

Results

(a) Histidinol dehydrogenase

Fig. 2.7 shows the sedimentation coefficient of histidinol dehydrogenase at concentrations of 0.65 - 8.7mg/ml in 0.1M sodium/potassium phosphate buffer, pH 6.8. The sedimentation coefficient increases with decreasing concentration, a characteristic of most macromolecules (Schachman, 1959b). Extrapolation to zero concentration gives a sedimentation coefficient of 6.3 - 6.4.¹ The sedimentation behaviour of the

¹

This value is not in agreement with the value of 7.1S reported by Creaser *et al.* (1967). The value of 7.1 was obtained in the Dept. of Microbiology, Birmingham University. The sedimentation coefficients of preparations of histidinol dehydrogenase obtained at the A.N.U. over a three year period were always in agreement with the values given in fig. 2.7. It appears that preparations of histidinol dehydrogenase obtained

(Continued p.108)

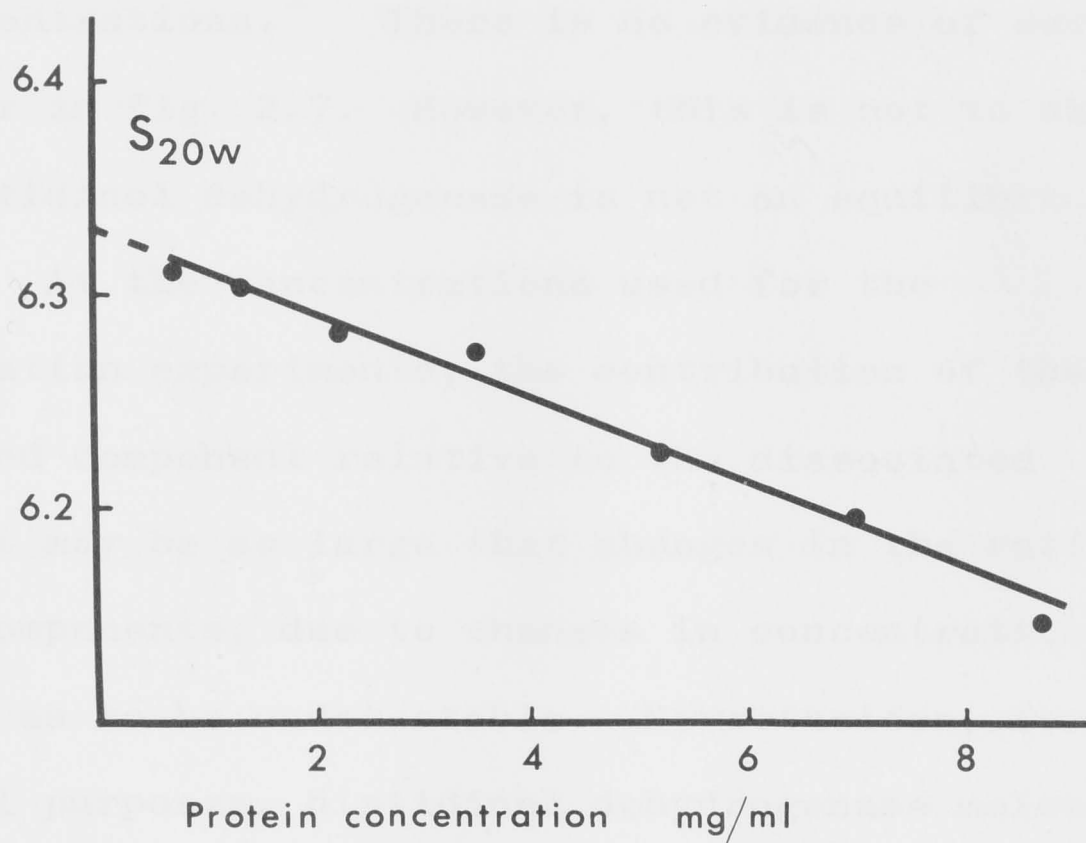


Figure 2.7 Effect of concentration on the sedimentation coefficient of histidinol dehydrogenase

protein in this concentration range is typical of a preparation which is homogeneous with respect to molecular weight. Equilibrium mixtures of molecules with different molecular weights show a decrease in sedimentation coefficient with decreasing concentration, because the contribution of the dissociated component increases at low concentrations.¹ There is no evidence of such behaviour in fig. 2.7. However, this is not to say that histidinol dehydrogenase is not an equilibrium mixture. At the concentrations used for the sedimentation experiments, the contribution of the associated component relative to the dissociated component may be so large that changes in the ratio of the components, due to changes in concentration, are so small as to be undetectable. Nevertheless, for practical purposes, histidinol dehydrogenase molecules in the concentration range 1 - 8mg/ml may be assumed to be homogeneous with respect to molecular weight.

¹ (continued from p.107)

in Birmingham were physically different from those obtained in Canberra, and this is presumably due to some difference in the growth of the organism or purification of the protein in the two laboratories.

¹

A good example of the concentration dependent sedimentation coefficient of a protein exhibiting association-dissociation equilibria is given by the sedimentation behaviour of α -chymotrypsin (Schwert, 1949).

Table 6.7 gives molecular weight estimates of a preparation of histidinol dehydrogenase of concentration 6.4mg/ml in 0.1M sodium/potassium phosphate buffer, pH 6.8. The apparent slight increase in molecular weight with time is not significant. Between the first and last estimation the meniscus concentration falls from 5.2mg/ml to 3.8mg/ml, and since the observed molecular weight does not decrease during the course of the experiment, the molecular weight is not concentration dependent in this concentration range, a result already predicted from the sedimentation behaviour of the protein. The mean value of the estimates given is 122,800, but a safer estimate of the molecular weight would be 115 - 130,000.

In summary, histidinol dehydrogenase in the concentration range 1 - 8mg/ml in 0.1M sodium/potassium phosphate buffer, pH 6.8 has a molecular weight of approximately 122,000, and an S_{20w} of 6.15 - 6.3.

(b) PRAMP cyclohydrolase

Unfortunately, insufficient quantities of PRAMP cyclohydrolase were available to allow sedimentation coefficients to be estimated over as wide a range of concentrations as was possible for histidinol dehydrogenase. However, estimations at 1mg/ml,

Table 6.7 Estimations of the molecular weight of
histidinol dehydrogenase

Time after rotor reached speed	Estimated M.W.	Average	Concentration at meniscus
16min	123,600 117,200 118,300	119,400	5.2mg/ml
64min	123,500 122,400	123,000	
112min	123,200 127,500 121,300	123,000	
160min	124,800 125,900	125,000	3.8mg/ml
Mean value	122,800		

Estimations were performed on a 0.64% protein
solution in 0.1M sodium/potassium phosphate, pH 6.8.
Rotor speed = 9945rpm Temperature = 21^o.

Different values for the same time are estimates
made from independent series of measurements from
the same photograph.

1.5mg/ml and 2.75mg/ml gave S_{20w} values between 7.45 and 7.55. This value is considerably higher than those obtained for histidinol dehydrogenase at the same concentrations, although the conditions used were identical for both sets of determinations.

Table 7.7 gives molecular weight estimations made on a PRAMP cyclohydrolase preparation of concentration 2.75mg/ml in 0.1M sodium/potassium phosphate buffer, pH 6.8. There was no significant variation in the observed molecular weight at the meniscus during the course of the experiment. The concentration at the meniscus between the first and last estimation fell from 2.1 to 1.3mg/ml, and this suggests that the average molecular weight of PRAMP cyclohydrolase preparations is not markedly concentration dependent in this concentration range. Previous experiments (see 'purity criteria' Chapter 6) have demonstrated, however, that preparations of PRAMP cyclohydrolase are equilibrium mixtures, the preparations showing considerable polydispersity at low concentrations. It follows that at the high concentrations used for the sedimentation and approach to equilibrium experiments, the equilibrium strongly favours one of the components of the mixture. The mean value of the ten estimations

Table 7.7 Estimations of the molecular weight of
PRAMP cyclohydrolase

Time after rotor reached speed	Estimated M.W.	Average	Concentration at meniscus
12min	119,400 122,200	120,800	2.1mg/ml
28min	127,300 133,300	130,300	
60min	127,800 131,200	129,000	
92min	121,200 126,400	123,800	
134min	124,800 129,600	127,200	1.3mg/ml
Mean Value	126,300		

Estimations were performed on a 0.25% protein
solution in 0.1M sodium/potassium phosphate,
pH 6.8. Rotor speed = 13140rpm. Temperature
= 21⁰.

given is 126,300, a value only about 3% different from that obtained for histidinol dehydrogenase. It is apparent from the range of values obtained for both proteins that this is not a significant difference.

Discussion

Taken in combination with the indirect evidence discussed at the beginning of this chapter, the experiments performed here provide strong evidence that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical proteins. Preparations of the two proteins have very similar amino acid compositions, and, in the concentration range where each protein appears to be homogeneous with respect to molecular weight, the same molecular weight.

The difference in the sedimentation coefficients of the two proteins may be a reflection of the physical differences which result in one protein being uni-functional and the other tri-functional. Since both proteins have the same molecular weight at this concentration, they must contain the same number of sub-units, and the difference in sedimentation coefficients must result from a difference in the frictional coefficient due to differences in the arrangement of these sub-units.

The concentrations at which these observations were made are several orders of magnitude higher than the concentrations of enzyme normally used under assay conditions. Thus the differences in catalytic properties need not result directly from the differences in conformation observed at these high concentrations. It is apparent that PRAMP cyclohydrolase, at low concentrations, is capable of dissociating into smaller molecular weight units, and it may be that the conformation of histidinol dehydrogenase is such as to prevent similar dissociation.

Minimum chemical molecular weight and sub-unit structure

Since PRAMP cyclohydrolase is capable of catalysing three quite different biochemical reactions, it is of central importance to find whether this protein is composed of one type of polypeptide, or is a heteropolymer composed of two or more types of polypeptide. Experiments have not been performed to establish the sub-unit structure of PRAMP cyclohydrolase, largely because of the difficulties of obtaining sufficiently large quantities of the purified protein. However, there is strong evidence that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical, so

that we may attempt to infer the structure of the former protein from a knowledge of the latter.

The amino acid analyses of PRAMP cyclohydrolase gave a good fit to an integer composition of 132 - 133 residues with a total weight of about 14,500. Histidinol dehydrogenase was found to have a very similar composition. Reference to table 4.7 shows that the integer composition of PRAMP cyclohydrolase contains 9 lysine and 5 arginine residues, while that of histidinol dehydrogenase contains 9 lysine and 4 arginine residues. Since trypsin cleaves polypeptide chains at arginine and lysine residues with high specificity (Desnuelle, 1960), it follows that if the amino acid composition of 132 - 133 residues represents the true chemical unit, then digestion with trypsin should yield approximately 15 peptides. Bennett and Creaser (1967) reported that tryptic digests of performically oxidised histidinol dehydrogenase yielded 14 ninhydrin positive spots after paper electrophoretic and chromatographic separation. This observation fits well with the amino acid composition given in table 4.7, and adds weight to the hypothesis that this amino acid composition represents the minimum chemical unit.

(1968) found that leucine is the only C-terminal residue

Does the minimum chemical unit consist of a single polypeptide of molecular weight 14,500, or is it composed of different polypeptides with this aggregate molecular weight? Attempts to detect N-terminal amino acids in preparations of histidinol dehydrogenase have always yielded negative results, and it seems therefore, that the protein contains no free N-terminal amino acids (Bennett, Creaser and MacDonald, 1968). The failure to detect N-terminal residues led Bennett et al. (1968) to perform a C-terminal analysis of histidinol dehydrogenase. These authors found that leucine was liberated at a higher rate than any other amino acid by carboxypeptidase digestion, and they obtained a yield of one mole of leucine per 10,500gm of histidinol dehydrogenase, a result which indicates a molecular weight of approximately 10,000 rather than 14,500. If the minimum chemical molecular weight is taken as 14,500, then the high yield of leucine from carboxypeptidase digestion could be explained by the presence of a second leucine residue in the short sequence adjacent to the C-terminal.

The hydrazinolytic procedure yields the C-terminal amino acid only, and using this procedure Bennett et al. (1968) found that leucine is the only C-terminal residue

in histidinol dehydrogenase. The yield of leucine was one mole per 16,000gm of protein, and this would represent a yield of 90% from a polypeptide of molecular weight 14,500. Yields of C-terminal residues from hydrazinolysis are known to vary considerably from one protein to another, and from one preparation of hydrazine to another, but yields as high as 90% are very rarely quoted in the literature (Braun and Schroeder, 1967), so that this result also suggests a polypeptide of molecular weight less than 14,500.

Bennett et al (1968) interpreted their results to mean that histidinol dehydrogenase contained two types of sub-unit, each with a C-terminal leucine residue, and with a combined molecular weight of 20,000. This interpretation was based on previous evidence that the protein had a minimum chemical molecular weight of 20,000 (Creaser, Minson and Bennett, 1967). This minimum chemical molecular weight was estimated from the results of amino acid analyses which were performed using a long path-length colorimeter cell in the amino acid analyser. As a result these early analyses were more sensitive by a factor of 10 than those described in this work. Although the use of the long path-length cell reduces

only slightly the accuracy of determinations of the acidic and neutral amino acids, it has become apparent that considerable errors may be introduced into the estimation of the basic amino acids. The greater imprecision of the original estimations of lysine and arginine may have led, if low, to a high estimation of the minimum chemical molecular weight when the amino acid analyses were correlated with the observed number of tryptic peptides. On the basis of the amino acid composition reported in this work, histidinol dehydrogenase has a minimum chemical molecular weight considerably lower than 20,000, and it is not necessary, therefore, to interpret the results of the C-terminal analysis in terms of two dissimilar sub-units.

The discrepancy between the minimum chemical weight estimated here, and the results of the C-terminal analysis performed by Bennett et al. (1968), although not completely irreconcilable, is sufficiently serious to prevent any decision as to the sub-unit structure of histidinol dehydrogenase. It should perhaps be stressed that the proposed minimum chemical molecular weight of 14,500 is not a measured quantity. It has been inferred from the amino acid analyses, and is

supported by the observed number of tryptic peptides. There are good grounds for believing that the value of 14,500 is more reliable than the previous estimate of 20,000. Nevertheless, it is clearly essential to obtain a more direct estimate of the minimum chemical molecular weight. Work is now in progress to separate tryptic peptides of histidinol dehydrogenase by ion exchange chromatography. This work has been undertaken by Dr D.J. Bennett, and will allow a quantitative analysis of all the components of tryptic digests. This should give a direct determination of the amino acid composition of the minimum chemical unit, and hence a direct estimation of the minimum chemical molecular weight. When correlated with the results of the C-terminal analysis, this should allow an unambiguous description of the sub-unit structure.

CHAPTER 8

General DiscussionPRAMP cyclohydrolase and the his-3 locus

The his-3 locus of Neurospora crassa specifies a protein which catalyses three reactions, PRATP \rightarrow PRAMP, PRAMP \rightarrow BBM II and histidinol \rightarrow histidine. The protein is polymeric, but the presently available chemical evidence does not allow a decision as to whether the subunits are identical or not. The genetic evidence also is ambiguous in this respect, but suggests the existence of non-identical sub-units.

The fact that the sequential reactions PRATP \rightarrow PRAMP and PRAMP \rightarrow BBM II are catalysed by a single protein casts some doubt on the status of PRAMP as a free intermediate in the histidine pathway of *Neurospora*. A weakness of this work has been that quantitative measurements of the independent reactions PRATP \rightarrow PRAMP and PRAMP \rightarrow BBM II have not been made. In fact the use of the assay of the overall reaction PRATP \rightarrow BBM II has been justified by the results obtained. Loss of pyrophosphohydrolase activity was always accompanied by loss of cyclohydrolase activity and vice versa, but the loss of these activities was

not necessarily accompanied by loss of histidinol dehydrogenase. Similarly, several techniques were found to result in the loss of histidinol dehydrogenase activity but not of pyrophosphohydrolase and cyclohydrolase activities. It can be envisaged that the protein may be nominally divided into two parts, one being essential to histidinol dehydrogenase activity and the other part to the other two activities. It is tempting to speculate further that the pyrophosphohydrolase and cyclohydrolase reactions occur at the same catalytic site. Although the two reactions are chemically different, the two substrates PRATP and PRAMP are structurally very similar. However, if both reactions occurred at the same catalytic site, it would be reasonable to predict that a large class of mutants would be found which lacked both pyrophosphohydrolase and cyclohydrolase activities, but retained histidinol dehydrogenase activity. That this is not the case is apparent from the data of Ahmed et al. (1964). With one possible exception (589), his-3 mutants lack only one or all three enzyme activities, and this is consistent with the idea of three different catalytic sites. It is remarkable that a result that can be readily achieved by chemical manipulation, namely the

loss of both pyrophosphohydrolase and cyclohydrolase activities but the retention of histidinol dehydrogenase, is very rarely accomplished by mutation.

PRATP pyrophosphohydrolase, PRAMP cyclohydrolase and histidinol dehydrogenase in other organisms

The genes and enzymes of the histidine pathway have been studied in a number of organisms. In two organisms, Aspergillus nidulans (Berlyn, 1967) and Saccharomyces cerevisiae (Fink, 1964, 1966) PRATP pyrophosphohydrolase, PRAMP cyclohydrolase and histidinol dehydrogenase are specified by a single genetic locus, as is the case for *Neurospora*. It seems likely that the gene-enzyme relationship is identical in these three organisms, and that in each case the three enzyme activities are carried by a single protein. Some reservation should perhaps be made in the case of *Saccharomyces*. Fine structure mapping of the his-4 locus (Fink, 1966) has shown it to be very similar to the his-3 locus of *Neurospora* in that: (1) mutations which result in the loss of particular functions are localised on the genetic map and (2) the effects of mutation are polarised. In the case of his-3 mutants various exceptions to these generalisations were found both by Catchside (1965) and by Ahmed et al. (1964).

No such exceptions were found among mutants of *Saccharomyces*. Indeed, the genetic evidence is strong that the his-4 locus consists of three structural genes, and it may be that in *Saccharomyces* the three reactions can be catalysed by proteins of independent function.

Gene-enzyme relationships in the histidine pathway have been the object of intensive study in Salmonella typhimurium (Hartman, Loper and Serman, 1960; Hartman, Hartman and Serman, 1960; Ames, Garry and Herzenberg, 1960). In this organism the genes controlling histidine biosynthesis are arranged in an operon (Ames and Hartman, 1962, 1963), and the gene which specifies histidinol dehydrogenase is located at one end of the operon, while the genes specifying PRATP pyrophosphohydrolase and PRAMP cyclohydrolase are located at the other end (Loper et al. 1964). Histidinol dehydrogenase from this organism can be readily separated from the other two enzyme activities (Whitfield, Smith and Ames, 1964), and the protein has been purified (Loper and Adams, 1965; Loper, 1968; Yourno and Ino, 1968). Mutants lacking histidinol dehydrogenase are separated on the genetic map from mutants lacking the other two functions in Staphylococcus aureus (Kloos and Pattee, 1965a,b) and Streptomyces

coelicolor (Russi, Carere, Fratello and Khoudokormoff, 1966), and it seems likely that histidinol dehydrogenase is a unifunctional protein in these organisms also.

The situation with respect to PRATP pyrophosphohydrolase and PRAMP cyclohydrolase is less clear. It is notable that in all cases where mutants lacking each activity have been identified (*Neurospora*, *Aspergillus*, Yeast and *Salmonella*), the mutants are closely linked. In *Salmonella* the reactions are catalysed by proteins specified by two contiguous genes, his-I and his-E, of the histidine operon. These two enzymes have not been studied in detail in *Salmonella*, but Whitfield et al. (1964) have estimated the sedimentation coefficient of both enzymes by the sucrose density gradient method of Martin and Ames (1961). Sedimentation coefficients of 3.4, 3.6 and 3.5 were obtained for PRATP pyrophosphohydrolase, and 3.6, 3.8 and 4.0 for PRAMP cyclohydrolase. The observed difference in the sedimentation coefficients of the two activities may not be significant, especially in view of the difficulties of measuring the pyrophosphohydrolase reaction (Smith and Ames, 1965), and it may be that these activities are carried by the same protein.

In summary, histidinol dehydrogenase is a unifunctional enzyme in *Salmonella*, and probably also in *Staphylococcus* and *Streptomyces*, but is associated with PRATP pyrophosphohydrolase and PRAMP cyclohydrolase activities in *Neurospora* and probably in *Aspergillus* and possibly in *Yeast*. Whether the pyrophosphohydrolase and cyclohydrolase reactions are catalysed by the same protein, or by different proteins, in *Salmonella*, *Staphylococcus* and *Streptomyces* is, as yet, unknown. Finally, preliminary information is available in *Pseudomonas aeruginosa* (Mee and Lee, 1967). Histidine mutants of this organism can be divided into five groups on the basis of linkage studies, and mutants of at least two of these groups are heterogeneous with respect to their biochemical characteristics. One of these heterogeneous groups contains mutants which lack histidinol dehydrogenase.

When more information is available, it should be possible to obtain an insight into the evolutionary relationship between the proteins which catalyse these three reactions in different organisms.

Multi-functional enzymes and enzyme aggregates

Whether an enzyme is to be defined as multi-functional or uni-functional depends in many cases on deciding whether particular biochemical events should

properly be regarded as one enzymic function or more than one. Many ambiguous situations exist. The problem is illustrated by the case of histidinol dehydrogenase. The oxidation of histidinol to histidine involves the NAD linked removal of two protons, and the oxidation of the amino alcohol to the amino acid apparently proceeds through the amino aldehyde intermediate, histidinal (Adams, 1955). Mechanistically, this may be regarded as two reactions. An ambiguous situation also exists where a particular enzyme is not completely specific with respect to its substrates. Chymotrypsin, for instance, is capable of hydrolysing peptide bonds between a wide range of amino acids. This enzyme could be said to be capable to catalysing many different reactions. This discussion will attempt to avoid situations of this sort, but it is apparent that in some instances the definition multi-functional or uni-functional is an ambiguous one.

In the past few years a number of proteins with multiple catalytic functions have been recognised. A comparison of some well established examples is made in table 1.8. These examples show a very wide range of structural and functional complexity. Fatty acid synthetase, which catalyses repeating rounds of

Table 1.8 A comparison of the properties of some multi-functional enzymes.

Name and Reactions Catalysed	Relationship Between Reactions Catalysed	Organism	Nature of Evidence for Multi-functional Protein	Properties	References
'Fatty acid synthetase' Acetyl-CoA:ACP S-acetyltransferase Malonyl-CoA:ACP S-malonyltransferase Acyl-ACP:malonyl-ACP ligase β -ketoacyl-ACP:NADPH oxidoreductase D- β -hydroxyacyl-ACP hydrolyase α,β -dehydroacyl-ACP:NADPH oxidoreductase (ACP = acyl carrier protein)	Sequential reactions in the de novo synthesis of long chain fatty-acids from: Acetyl-CoA + (Malonyl-CoA) _n	Yeast	Enzyme purified	M.W. = 2.3×10^6 Seven different types of sub-unit Enzyme from <u>E.coli</u> is separable into units of independent function	Lynen, 1961 Reviews: Vagelos, 1964 Olsen, 1966
L-histidinol phosphate phosphohydrolase (E.C.3.1.3.15) + D-erythro imidazoleglycerol phosphate hydrolyase (E.C.4.2.1.19)	Seventh and ninth reactions in histidine biosynthesis.	<u>Salmonella typhimurium</u>	Genetic evidence Co-fractionation of enzyme activities.	Different catalytic sites. Genetic evidence suggests homopolymer. M.W.s 145,000 and 75,000 observed.	Loper, 1961 Loper <u>et al</u> , 1964 Vasington and LeBeau, 1967
Homoserine:NAD oxidoreductase (E.C.1.1.1.3) + ATP:L-aspartate 4-phosphotransferase (E.C.2.7.2.4)	First and third reactions in the conversion of aspartate to threonine	<u>E.coli</u>	Genetic evidence. Protein partially purified.	M.W. approx. 180,000. Both activities inhibited by threonine	Cohen, Patte and Truffa-Bachi, 1965 Patte, Truffa-Bachi and Cohen, 1966 Truffa-Bachi, LeBras and Cohen, 1966a,b
L-malate:NAD oxidoreductase (E.C.1.1.1.37) + L-aspartate:2-oxoglutarate amino-transferase. (E.C.2.6.1.1)	—	<u>N.crassa</u>	Genetic evidence. Co-fractionation of enzyme activities. Chemical identity of proteins carrying one or other activity.	S _{20w} = about 4.5 Two activities readily separable by certain fractionation methods.	Nunkres, 1965a,b Kitto <u>et al</u> , 1967
meso-tartaric acid dehydrogenase + Dihydroxyfumaric acid reductive decarboxylase.	First and second steps in the metabolism of meso-tartaric acid.	<u>Pseudomonas acidovorans</u>	Protein purified.	S _{20w} = 4.7	Kohn and Jacoby, 1966
Chorismate mutase T + prephenate dehydrogenase	First and second steps in the biosynthesis of tyrosine	<u>Aerobacter aerogenes</u> <u>E.coli</u>	Genetic evidence. Protein partially purified Genetic evidence Co-fractionation.	M.W. = approx. 100,000. Dissociates to units of about 50,000. —	Cotton and Gibson, 1965, 1967, 1968

Table 1.8 continued

Name and Enzymic Functions	Relationship Between Reactions Catalysed	Organism	Nature of Evidence for Multi-functional Protein	Properties	References
Chorismate mutase P + Prephenate dehydratase	First and second steps in the biosynthesis of phenylalanine.	<u>Aerobacter aerogenes</u> <u>E. coli</u>	Genetic evidence. Co-fractionation of enzyme activities.	—	Cotton and Gibson, 1965
N-(5'-phosphoribosyl) anthranilate isomerase + Indoleglycerol phosphate synthetase	Third and fourth steps in the biosynthesis of tryptophan.	<u>E. coli</u> <u>Salmonella typhimurium</u>	Protein purified. Genetic evidence	M.W. = 50,000 Monomer. —	Creighton and Ianofsky, 1966 Blume and Balbinder, 1966
Anthranilate synthetase + N-(5'-phosphoribosyl) anthranilate isomerase + Indoleglycerol phosphate synthetase	First, third and fourth steps in the biosynthesis of tryptophan.	<u>N. crassa</u> <u>Aspergillus nidulans</u>	Genetic evidence. Co-fractionation of enzyme activities. Co-fractionation of enzyme activities.	M.W. = 200,000 Heteropolymer, since specified by unlinked loci. —	Wegman and DeMoss, 1965 DeMoss and Wegman, 1965 Hutter and DeMoss, 1967a Hutter and DeMoss, 1967a,b
Anthranilate synthetase + Anthranilate-5-phosphoribosyl: phosphoribosyltransferase	First and second steps in the biosynthesis of tryptophan.	<u>E. coli</u> <u>S. typhimurium</u> <u>Aerobacter aerogenes</u>	Genetic evidence. Co-fractionation of enzyme activities. Protein partially purified.	Genetic evidence suggests heteropolymer —	Ito and Ianofsky, 1966 Bauerle and Margolin, 1966a,b Egan and Gibson, 1966
3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (DAHP synthetase) + Chorismate mutase	First reaction in the common aromatic pathway + First reaction in the biosynthesis of tyrosine and phenylalanine.	<u>Bacillus subtilis</u>	Genetic evidence. Co-fractionation of enzyme activities.	M.W. = approx. 150,000 Genetic evidence suggests heteropolymer. May also carry shikimate kinase activity.	Nester, Lawrence and Nasser, 1967
5-dehydroquinate synthetase. + 5-dehydroquinate hydrolase (E.C.4.2.1.10) + Shikimate:NADP oxidoreductase (E.C.1.1.1.25) + Shikimate kinase + 3-enolpyruvylshikimate synthetase	Reactions 2 - 5 inclusive in the common aromatic pathway.	<u>N. crassa</u>	Genetic evidence Co-fractionation of enzyme activities	M.W. = approx. 200,000 Genetic evidence suggests several types of sub-unit. .	Giles, Case, Partridge and Ahmed, 1967

condensation, reduction and dehydration, has a molecular weight of 2.3×10^6 , and is composed of seven types of sub-unit. This represents a very high level of molecular organisation. The indoleglycerol phosphate synthetase of E. coli, on the other hand, catalyses two sequential reactions and is composed of a single sub-unit of molecular weight 50,000. Direct evidence concerning sub-unit structure is available in only a few cases, but on the basis of indirect evidence many of the proteins are heteropolymeric and a few are homopolymeric. The relationship between the reactions catalysed also shows considerable variation. In some cases the protein catalyses sequential reactions and in others non-sequential reactions in a particular biochemical pathway. In one case, aspartate amino transferase / malate dehydrogenase, there is no obvious relationship between the two reactions. It should be pointed out that, in this latter case, the work of Kitto et al. (1967) casts some doubt on the conclusion of Munkres (1965a,b) that these two activities are carried by a single protein. However, Munkres' evidence is strong, and the best interpretation of Kitto's results is that the bi-functional protein can, under certain circumstances, be fractionated into units of independent function. The

observation that most multi-functional proteins catalyse related biochemical reactions may be a selection artefact, because of the low probability of recognising, by chance, an enzyme which catalyses unrelated reactions.

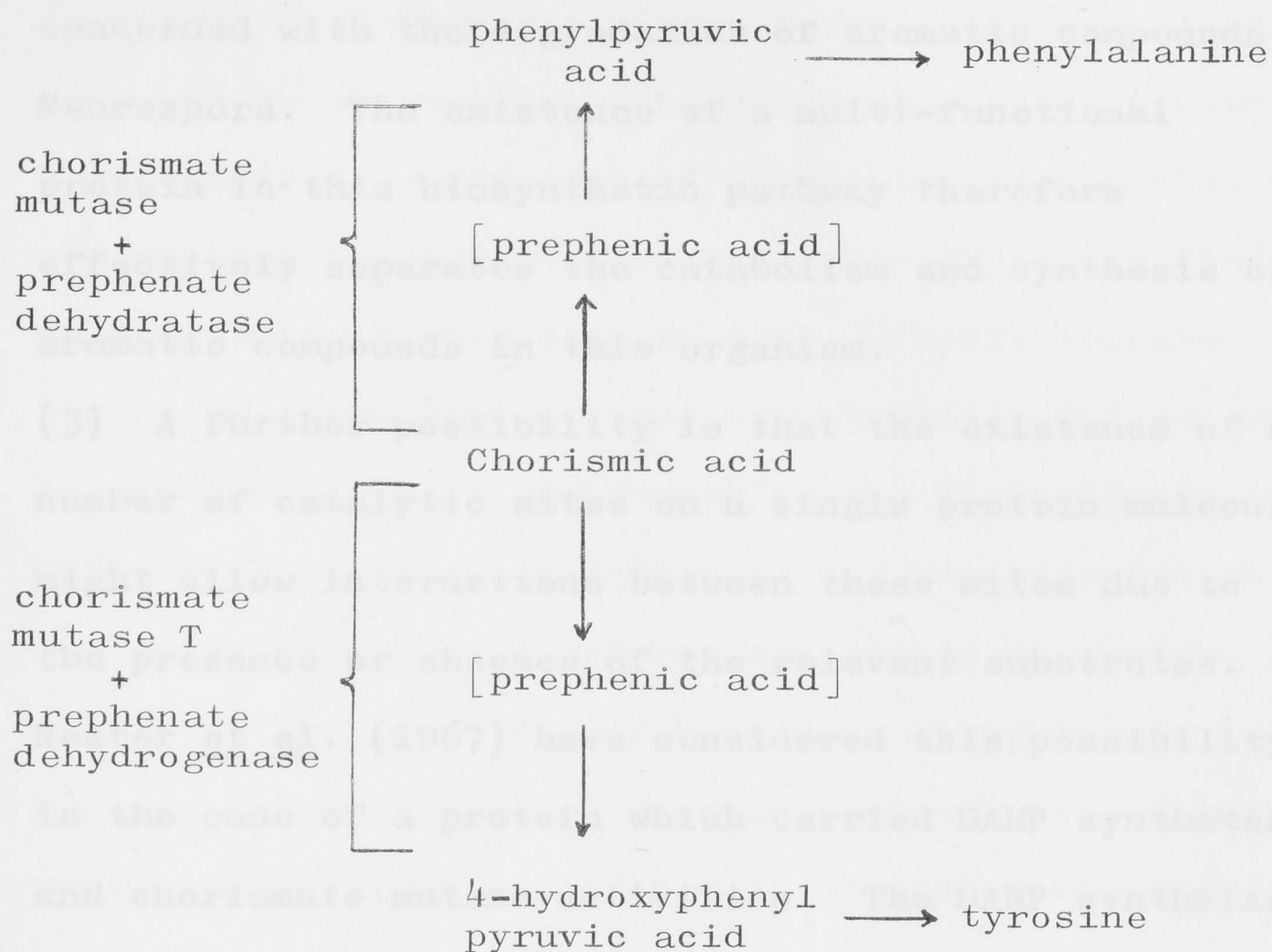
The role of multi-functional proteins

It is often possible to rationalise the existence of a particular multi-functional protein by suggesting advantages of the multi-functional enzyme over an equivalent mixture of uni-functional enzymes. A number of possible advantages can be suggested.

(1) Where a protein catalyses a series of sequential reactions, it can be argued that such an enzyme has an advantage over a series of uni-functional enzymes in that the intermediates of the pathway are localised in close proximity to the catalytic sites which act upon them. Such arguments are particularly convincing when it can be shown that the intermediates remain enzyme-bound throughout the series of reactions. This argument is attractive in that it helps to destroy the idea that the cell is a 'soup' of enzymes and intermediates, a concept which most biologists find unsatisfactory. This explanation does not account for the existence of enzymes which catalyse non-sequential reactions in a particular biochemical pathway, but the

problem here is that such enzymes are studied in vitro. A protein which is known to catalyse reactions 2, 3 and 6 of a reaction sequence in vitro, may in vivo be associated with proteins which catalyse reactions 1, 4 and 5. The recognition of such a situation would prove very difficult.

(2) A special case of (1) is where a particular intermediate exists in two different biochemical pathways. If the intermediate is both the substrate and the product of a multi-functional protein, and if it remains enzyme-bound, then the intermediate is restricted to one pathway, and cannot be metabolised by the other. This might be of particular importance where the two pathways are under the control of different factors. Cotton and Gibson (1965) proposed the following scheme to explain the existence of the chorismate mutase P / prephenate dehydratase and chorismate mutase T / prephenate dehydrogenase proteins.



According to this scheme, prephenic acid is not a shared intermediate in the tyrosine and phenylalanine pathways. A similar situation was recognised by Giles, Partridge, Ahmed and Case (1967). Neurospora crassa was found to contain two forms of 5-dehydroquinate hydrolase. One form is associated with a multi-functional protein which catalyses reactions 2-5 of the common aromatic pathway (see table 1.8), and the other form has different physical properties and is inducible by quinic acid. It appears that the inducible form is

concerned with the degradation of aromatic compounds in *Neurospora*. The existence of a multi-functional protein in this biosynthetic pathway therefore effectively separates the catabolism and synthesis of aromatic compounds in this organism.

(3) A further possibility is that the existence of a number of catalytic sites on a single protein molecule might allow interactions between these sites due to the presence or absence of the relevant substrates. Nester et al. (1967) have considered this possibility in the case of a protein which carried DAHP synthetase and chorismate mutase activities. The DAHP synthetase reaction is inhibited both by the substrate, chorismic acid, and by the product, prephenic acid, of the other enzyme activity, chorismate mutase. The possibility exists that the catalytic site for chorismate mutase is the allosteric site for DAHP synthetase. Such a situation would achieve a delicate balance between the production and metabolism of common aromatic pathway intermediates.

The role of the tri-functional protein PRAMP cyclohydrolase

It is notable that of the multi-functional proteins which have been recognised to date, the majority are

concerned with aromatic biosynthesis. Although this is no doubt partly due to the considerable attention which has been paid to aromatic biosynthesis, it can also be argued that it would be reasonable to expect to find multi-functional proteins in this pathway, because the pathway contains several branch points and several intermediates which are common to the biosynthesis of different end products. This is not the case for the histidine pathway. There are apparently no branch points in the ten steps of histidine biosynthesis, because *Salmonella* mutants which lack all ten enzymes grow normally when supplied with histidine (Ames et al., 1967).

It is difficult to rationalise the existence of a protein which catalyses reactions 2, 3 and 10 in the histidine pathway of *Neurospora*. The possibility cannot be ignored that, in vivo, all ten reactions in the pathway are catalysed by a large enzyme aggregate. However, this seems unlikely. Nor can we rule out the possibility of a control mechanism associated with this protein, but histidine or histidinol at concentrations of 2mM have no detectable effect on the catalysis of the reaction $\text{PRATP} \rightarrow \text{PRAMP} \rightarrow \text{BBM II}$. Furthermore, any conceivable control mechanism applied to reactions 2,

3 or 10 would be irrelevant, because the first enzyme in the pathway is inhibited by histidine (see Appendix III).

With regard to the catalysis of single reactions, multi-functional proteins must be less efficient than their uni-functional counterparts, because the structure of a multi-functional protein is compromised by the fact that the protein must catalyse a number of different reactions. Thus, an alteration which improves the efficiency of one enzyme function might decrease the efficiency of a second function, and the inflexibility imposed by such a situation increases with the number of reactions catalysed. This reasoning suggests that, all other factors being equal, the evolution of proteins should be towards the division of labour rather than the sharing of labour. Discussions of multi-functional proteins have often emphasised the evolution of these proteins from uni-functional proteins (Bonner, DeMoss and Mills, 1965; DeMoss, 1965; Hutter and DeMoss, 1967a), but it is apparent that evolution in the reverse direction is equally probable. I have argued that there is no conceivable advantage in reactions 2, 3 and 10 of the histidine pathway being catalysed by a single protein. The PRAMP cyclohydrolase

of *Neurospora* might therefore be considered a 'primitive' protein when compared with the 'specialised' proteins which catalyse the same reactions in *Salmonella*.

From the presently available information it is clear that the histidinol dehydrogenase of *Salmonella* is structurally very different from the tri-functional enzyme which exists in *Neurospora*. Histidinol dehydrogenase from *Salmonella* consists of two identical sub-units of molecular weight 40,000 (Loper, 1968; Yourno, 1968). The sub-unit structure of the *Neurospora* enzyme is not yet known, but the sub-units cannot be larger than 14,000 molecular weight, and may be smaller than this. The evolutionary relationship between these two proteins is of considerable interest, but this will not be resolved until the amino acid sequences of the tri-functional protein in *Neurospora* and the corresponding proteins in *Salmonella* have been elucidated.

the association and dissociation of the sub-units of this protein. The weight-average molecular weight of preparations of PRMP cyclohydrolase will be determined at low concentrations by boundary elution from Sephadex gels. The sedimentation coefficients of the protein over the concentration range 1 - 10mg/ml will be determined when sufficient pure protein has been obtained.

Suggestions for further work

The work described in this thesis cannot be regarded as completed. The following investigations will take priority in further work.

(1) It is essential that the sub-unit structure of PRAMP cyclohydrolase be determined. It is expected that the sub-unit structure of histidinol dehydrogenase will soon be known. I have presented evidence that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical, but this evidence is not completely conclusive and the sub-unit structure of PRAMP cyclohydrolase will have to be determined independently.

(2) Some of the physical properties of PRAMP cyclohydrolase at high concentrations have been determined. However, the protein is polydisperse at low concentrations, and it is important to investigate the association and dissociation of the sub-units of this protein. The weight-average molecular weight of preparations of PRAMP cyclohydrolase will be determined at low concentrations by boundary elution from Sephadex gels. The sedimentation coefficients of the protein over the concentration range 1 - 10mg/ml will be determined when sufficient pure protein has been obtained.

Of course, there are many other lines of investigation which might be undertaken. In theory, a pure protein with three catalytic activities is worthy of intensive study, but it is apparent that the low yields of pure protein which are obtained severely limit the amount of work which can be done. However, if, as expected, it can be shown conclusively that PRAMP cyclohydrolase and histidinol dehydrogenase are chemically identical, then information about the former protein can be obtained from chemical studies of the latter. This is a practical proposition because pure histidinol dehydrogenase is obtainable in much larger amounts.

An important line of enquiry is the nature of the proteins which catalyse reactions 2, 3 and 10 of the histidine pathway in other organisms. Of particular interest is the question whether PRATP pyrophosphohydrolase and PRAMP cyclohydrolase activities are carried by a single protein or by different proteins in *Salmonella*. It is hoped to answer this question, and to investigate the fractionation of PRATP pyrophosphohydrolase, PRAMP cyclohydrolase and histidinol dehydrogenase in extracts of other organisms.

APPENDIX I

Complementation and the interpretation of
complementation data

The genetic term 'complementation' refers to the ability of one mutant to compensate for the defect of another, and the phenomenon is studied by the introduction of two differently mutant genomes into a single unit of cytoplasm. In *Neurospora* this is achieved by the fusion of homocaryotic hyphae to form a stable heterocaryon.

Complementation can occur by three mechanisms.

(1) Mutual compensation through the production of intermediates.

If a mutant A is incapable of catalysing the reaction $(a) \rightarrow (b)$, and a mutant B is incapable of catalysing the reaction $(x) \rightarrow (y)$, then the heterocaryon A/B is able to grow because it is provided with intermediates (b) and (y). If $(a) \rightarrow (b)$ and $(x) \rightarrow (y)$ are catalysed by different proteins, then the heterocaryon has a 'wild type' protein content, and complementation has occurred between mutants of different structural genes (intra-allelic complementation). However, if the two reactions are normally catalysed by the same homopolymeric

protein, then mutations giving rise to A and B have occurred in the same structural gene, and complementation would be classified as inter-allelic.

(2) Restoration of function by re-assortment of 'wild type' protein sub-units.

Consider a reaction $(x) \rightarrow (y)$ catalysed by a heteropolymeric protein $(A'+B')_n$ specified by two structural genes A and B. Mutations in A or B may result in the loss of ability to catalyse $(x) \rightarrow (y)$. Thus A^-B^+ mutants and A^+B^- mutants cannot catalyse $(x) \rightarrow (y)$, and the two classes of mutants therefore have the same biochemical characteristics. The heterocaryon A^+B^-/A^-B^+ , however, catalyses the reaction $(x) \rightarrow (y)$, because the cell synthesises normal A' and B' subunits, and is therefore able to form the normal enzyme $(A'+B')_n$. Complementation has occurred between mutants of different structural genes and is intra-allelic. This kind of interaction is observed between some mutants of *Neurospora* which lack the enzyme anthranilate synthetase (Ahmad and Catcheside, 1960; DeMoss and Wegman, 1965).

(3) Mutual correction through the interaction of 'mutant' protein sub-units.

Complementation through this mechanism is usually referred to as inter-allelic complementation, although

complementation between allelic mutants may also occur through the mechanism described in (1) above. Inter-allelic complementation was first observed in *Neurospora* by Fincham and Pateman (1957), Giles, Partridge and Nelson (1957) and Catcheside and Overton (1958). Woodward (1959) showed that complementation occurred through the interaction of the two mutant proteins, and it was subsequently shown that this interaction resulted in the formation of a hybrid polymer from differently mutant sub-units (Fincham and Coddington, 1963; Coddington and Fincham, 1965). Thus the wild type homopolymer is active, the two mutant homopolymers are inactive, but the hybrid polymer formed in the heterocaryon is sufficiently active to promote growth. It appears that the co-operative effects involved in the association of protein sub-units results in a partial correction of the tertiary structure of the mutant polypeptides. This theory predicts that where the active form of the enzyme is a monomer, inter-allelic complementation of this type will not occur, and where it has been tested this prediction holds true (Margolies and Goldberger, 1966, 1967).

If complementation analysis is to be used to identify structural genes, and to predict possible

interactions between gene products, it is essential to distinguish between the mechanisms of complementation described in (1), (2) and (3). Given complementation between two mutants A and B, how may a decision be reached as to the mechanism by which complementation occurs?

Mechanism (1) may be easily distinguished from (2) and (3) because in this case A and B have different biochemical properties. Thus mutant A lacks a particular enzymic function and may accumulate the intermediate (a) while mutant B lacks a different function and may accumulate the intermediate (x).

Where complementation occurs through mechanism (2) the following characteristics are probable.

- (1) The heterocaryons grow as strongly as the wild type organism.
- (2) The enzyme formed in the heterocaryon is identical to the wild type enzyme, a fact which may be demonstrated by a comparison of the kinetic and heat inactivation properties.
- (3) Mutants A and B are not closely linked.
- (4) If further mutants with the same biochemical defects are obtained, all mutants will fall into A group or B group so that all mutants in one group will

complement with all mutants in the other. No class of non-complementing mutants will be found.

However, exceptions to these general statements may be found. Consider characteristic (2). The heterocaryon A^+B^-/A^-B^+ produces both normal and mutant forms of the two polypeptides A' and B' . The enzyme protein has the structure $(A'+B')_n$. Where $n=1$, four possible forms of the protein may exist in the heterocaryon: two parental types; the double mutant type and the wild type. The parental types are inactive by definition, and the double mutant type is presumably also inactive. The wild type protein is therefore the only active component in the heterocaryon, and enzyme activity in the heterocaryon therefore has the same characteristics as in the wild type organism. Where n is large, however, the number of possible combinations of mutant and normal sub-units is very large, and the probability of obtaining a protein consisting entirely of normal sub-units is very small. In this case the heterocaryon enzyme has different characteristics from that of the wild type organism. Furthermore, the heterocaryon may grow very slowly. This argument assumes that all combinations of sub-units occur with equal probability. In fact normal sub-units

probably associate with greater efficiency than do mutant sub-units. Nevertheless, it is clear that a wide range of growth responses, and of enzyme characteristics, may be observed in heterocaryons formed by this type of complementation.

Secondly, if the two structural genes are closely linked and are translated by a single messenger RNA molecule, characteristic (4) becomes invalid, because a mutation may alter the translation of both structural genes. In this case a mutant synthesises abnormal A' and B', and will not complement mutant A or mutant B.

Where complementation occurs through mechanism (3) the following predictions can be made.

- (1) That A and B are closely linked.
- (2) That the heterocaryon will grow less strongly than the wild type organism.
- (3) That if further mutants with the same biochemical defect are obtained, some will complement with A, and some will complement with B, but a large class of non-complementing mutants will also be found.
- (4) That the hybrid protein formed in the heterocaryon has different properties from the enzyme found in the wild type organism (Garen and Garen, 1963; Fincham, 1959).

Finally, if complementation is identified as being the result of mechanism (1), how can a decision be reached as to whether mutants A and B arise through mutations in the same or in different structural genes? If A and B are mutants of the same structural gene, the following predictions can be made.

- (1) That A and B are very closely linked.
- (2) That a class of mutants should be found which have lost both enzymic functions as a result of a single mutational event.
- (3) That mutant A (which lacks the ability to catalyse the reactions $(a) \rightarrow (b)$) should be altered in its ability to catalyse the reaction $(x) \rightarrow (y)$ (the enzymic function absent from mutant B). A comparison of the catalysis of the reaction $(x) \rightarrow (y)$ by the wild type enzyme and by the enzyme from mutant A should show some differences in the properties of the two enzymes.
- (4) The two enzymic functions should be inseparable by protein fractionation techniques.

It is apparent that a combination of linkage and preliminary biochemical data will usually permit an unambiguous decision as to the nature of complementation between particular groups of mutants. In complex

situations this may not be the case. Consider for example the situation where several biochemical reactions are catalysed by a single protein of structure $(A'+B')_n$, where A' and B' are different polypeptides specified by structural genes A and B , and where n is greater than one. Complementation between different mutants of A and B genes may occur by all of the mechanisms described above, and it is most unlikely that such a situation would be resolved without detailed examination of the protein.

APPENDIX II

Concerning the chemical comparison of histidinol
dehydrogenase and PRAMP cyclohydrolase

A comparison of the chemical composition of histidinol dehydrogenase and PRAMP cyclohydrolase was made by comparing amino acid analyses of the two proteins (see Chapter 7). Amino acid analysis is not the preferred technique for establishing the chemical identity or non-identity of two proteins. Purified preparations of many proteins contain a number of minor impurities, because low levels of impurities are very difficult to detect. Such impurities do not seriously interfere with chemical investigations such as C- or N-terminal analyses or peptide analysis, because under these conditions the effects of impurities can be recognised as such. Very low levels of terminal amino acids from a protein preparation can be assumed to be due to the presence of impurities and can be ignored. Furthermore, during terminal or peptide analyses, each impurity behaves independently. In the former case it is likely that each impurity will have a different terminal amino acid, and in the latter case each impurity will yield unique peptides. Total hydrolysis,

however, converts all the protein in a preparation to common units, thus destroying the unique properties of each protein in the preparation. The consequences of this are twofold. Firstly, the effects of impurities cannot be recognised as such. Secondly, since the impurities no longer have unique properties, they have a sum effect. Thus three contaminating species, each at a concentration of 3%, may have as large an effect as a single impurity at the 9% level. It is apparent, therefore, that two identical proteins may show small differences in amino acid composition if each or either protein is contaminated with low levels of different impurities.

Of the available methods for determining the identity or non-identity of two proteins, the obvious choice, for a number of reasons, is peptide comparison. The advantages of this method are as follows.

- (1) The technique is highly specific, and in many instances is capable of detecting single amino acid replacements, as originally demonstrated by Ingram (1958) using paper electrophoresis and chromatography procedures.
- (2) Peptides of minor impurities are very unlikely to be detected when the paper mapping technique is used,

so that minor impurities do not interfere with the comparison, even when several such impurities are present. However, although impurities are usually designated 'minor' or 'major' on a weight contribution basis, it is important to realise that it is the molar contribution which is important in the case of peptide mapping. An impurity of 10% by weight, but with a minimum molecular weight only 10% that of the major component, has the same molar concentration as the major component, and the peptides of such an impurity are, of course, readily detectable. The fact that tryptic peptide mapping observes differences at the molar level is pertinent to the comparison of histidinol dehydrogenase and PRAMP cyclohydrolase. It was pointed out in the introduction to Chapter 7 that any chemical differences between histidinol dehydrogenase and PRAMP cyclohydrolase would probably be due to the presence of an additional component in the latter protein. Consider, then, the situation where histidinol dehydrogenase is composed of one type of polypeptide $(A)_n$, and PRAMP cyclohydrolase two types of polypeptide $(A+B)_n$. If B were a very small polypeptide, say 10% of the molecular weight of A, then its effect on the amino acid analysis of A would be very small, and this

effect might be considered insignificant. Tryptic peptide mapping, however, should demonstrate unambiguously the presence of B in preparations of PRAMP cyclohydrolase, because B should yield a few additional peptides.

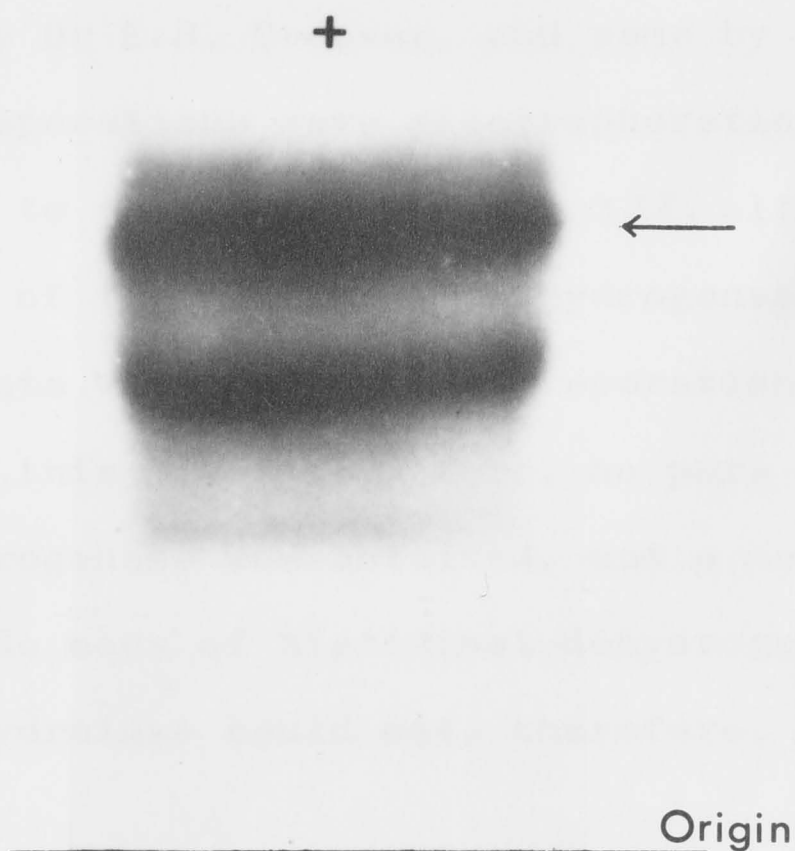
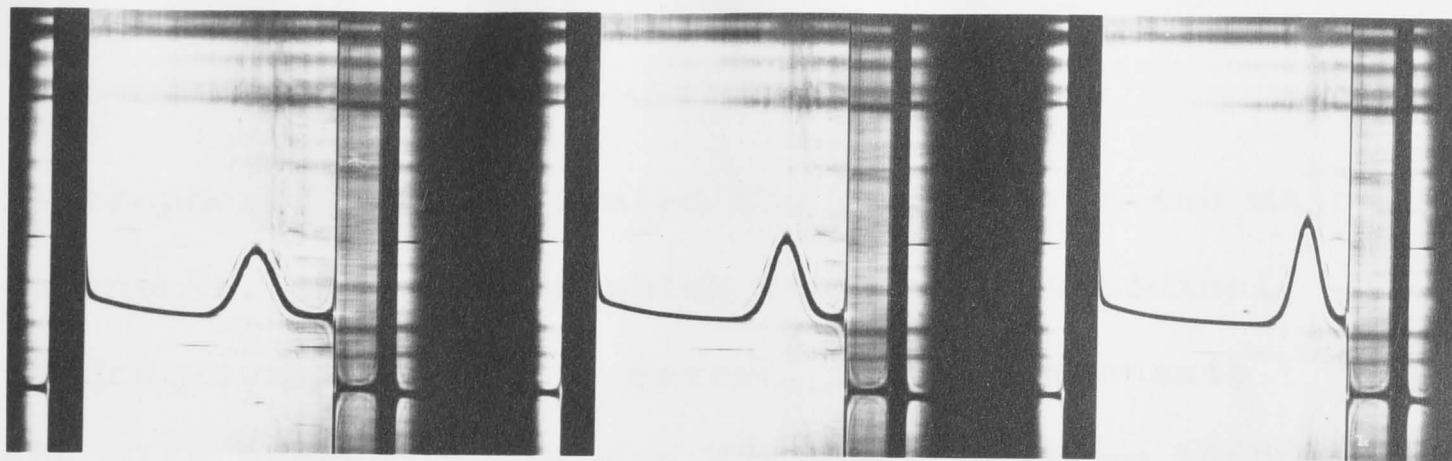
For these reasons a comparison of the chemical compositions of the two proteins was initially attempted using the technique of tryptic peptide mapping.

A disadvantage of paper peptide mapping is that the technique is not precisely reproducible, because of the variables involved in the separation, and to a lesser extent in the preparation, of peptides. For the precise comparison of two proteins it is therefore essential that the production and separation of peptides be performed, as far as possible, in parallel. Only under these experimental conditions can small differences in two peptide maps be regarded as meaningful (Claude Bennett, 1967). It is apparent from the comparison of the amino acid compositions of histidinol dehydrogenase and PRAMP cyclohydrolase (see Chapter 7), that any differences in the chemical composition of the two proteins are likely to be very

small. It follows that the experimental conditions described above are essential for a comparison of peptide maps of these two proteins.

A purified preparation of histidinol dehydrogenase was therefore obtained in order to compare tryptic peptide maps of the two proteins. Since large amounts of histidinol dehydrogenase were available relative to PRAMP cyclohydrolase (compare yields from purification of two proteins, tables 1.3 and 1.6), a number of peptide maps of histidinol dehydrogenase were first prepared in order to gain familiarity with the technique.

The results obtained were quite different from those reported by Bennett and Creaser (1967). The maps contained at least 40 ninhydrin positive spots, and a small amount of ninhydrin positive material remained at the origin. Since the methods of Bennett and Creaser (1967) had been repeated as precisely as possible, it seemed likely that the inconsistency in the results was due to some impurity in the histidinol dehydrogenase preparation. The preparation was therefore subjected to analytical ultracentrifugation and starch gel electrophoresis using the methods described in Chapter 6 under 'purity criteria'. The results are shown in figs. 1.II and 2.II. The protein sedimented as a single symmetrical boundary, but starch gel



Figures 1.II and 2.II Analytical ultracentrifugation and starch gel electrophoresis of a preparation of histidinol dehydrogenase purified in June 1968

Conditions of experiment were as described in Chapter 6 under 'purity criteria'.

- 1.II Analytical ultracentrifugation. The protein concentration is 6.2mg/ml. Sedimentation is from right to left.
- 2.II Starch gel electrophoresis. The gel has been stained for protein. The arrow indicates the position of a zone of histidinol dehydrogenase activity.

electrophoresis demonstrated the presence of two major components, only one of which contained histidinol dehydrogenase activity. Several minor components were also present. Between May and September 1968 several preparations of histidinol dehydrogenase were obtained (some purified by Dr E.H. Creaser, and some by this author). All preparations gave electrophoretic patterns similar to that shown in fig. 2.II, although the contribution of the histidinol dehydrogenase negative components varied from one preparation to another. During this time, therefore, no pure histidinol dehydrogenase was obtained, and a comparison of tryptic peptide maps of histidinol dehydrogenase and PRAMP cyclohydrolase could not, therefore, be made.

The question arises as to whether the impurities observed in these preparations are characteristic of all histidinol dehydrogenase preparations, or whether they are only present at high concentrations in preparations obtained after May 1968. The latter situation is established from the following facts. (1) Creaser et al. (1967) reported that purified histidinol dehydrogenase yielded a single protein zone after starch gel electrophoresis.

(2) Bennett and Creaser (1967) reported that tryptic digestion of histidinol dehydrogenase yielded 14 peptides (not 40 as observed by this author.)

(3) Bennett et al. (1968) reported that hydrazinolysis of histidinol dehydrogenase yielded significant amounts of only leucine. All other amino acids liberated by the hydrazinolysis procedure were at less than 5% the concentration of leucine. The preparations used for these experiments could not, therefore, have contained high levels of impurity.

The experiments with histidinol dehydrogenase described in Chapter 7 were carried out using protein prepared in January 1967. The protein was originally prepared for the purpose of investigating some of the physical properties of histidinol dehydrogenase (see Chapter 7), and a small amount remained which was later used to compare the amino acid composition of histidinol dehydrogenase and PRAMP cyclohydrolase. Unfortunately, insufficient material remained for a comparison of tryptic peptide maps. This preparation was subjected to analytical ultracentrifugation and starch gel electrophoresis, and the results are shown in figs. 3.II and 4.II. It is clear that this preparation is of much higher purity than that shown

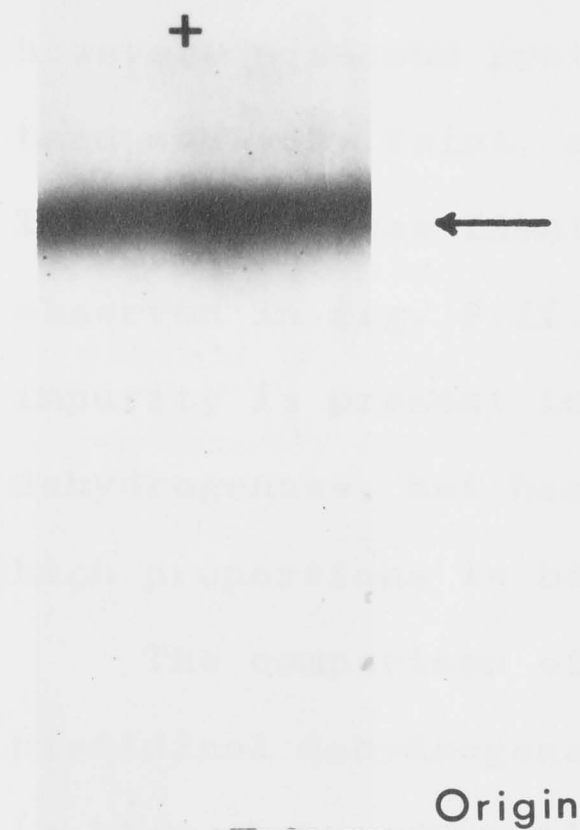


Figure 3.II

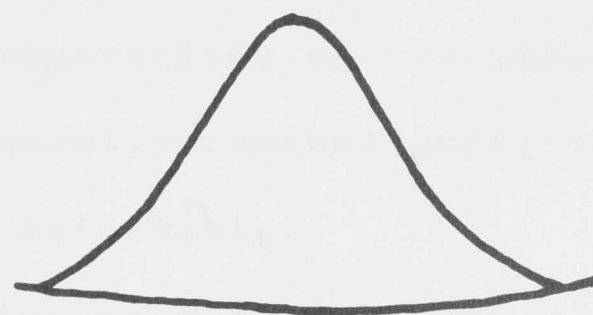


Figure 4.II

Figures 3.II and 4.II Analytical ultracentrifugation and starch gel electrophoresis of a preparation of histidinol dehydrogenase purified in January 1967.

Conditions of experiment were as described in Chapter 6 under 'purity criteria'.

3.II Starch gel electrophoresis. The gel has been stained for protein. The arrow indicates the position of a zone of histidinol dehydrogenase activity.

4.II Analytical ultracentrifugation. The protein concentration is 6.8mg/ml. The diagram shows a tracing of the Schlieren pattern obtained after centrifuging for 90min at 50740rpm.

in figs. 1.II and 2.II. After removal of all the excess stain from the starch gel by washing for several days, however, a second protein band was just detectable. This band was very faint, and is not visible in the photograph. Its position was identical to that of the major impurity observed in fig. 2.II. It seems, therefore, that this impurity is present in many preparations of histidinol dehydrogenase, but has only recently reached sufficiently high proportions to be easily detectable.

The comparison of the amino acid analyses of histidinol dehydrogenase and PRAMP cyclohydrolase (table 4.7) showed small differences in the amino acid composition of preparations of the two proteins. However, the histidinol dehydrogenase preparation used to make this comparison contained a detectable impurity, and the PRAMP cyclohydrolase preparation may have contained undetected impurities. These impurities, while insignificant for the purposes of most chemical and physical investigations, were probably sufficiently large to account for the observed differences in the amino acid compositions of the two preparations.

The causes of the large increase in levels of impurities in histidinol dehydrogenase prepared after May 1968 are unknown, but work is in progress to eliminate them.

APPENDIX III

Inhibition of the first enzyme in histidine biosynthesis in Neurospora

The possibility that the first enzyme in the histidine pathway of *Neurospora*, PRATP: phosphoribosyl transferase, is inhibited by histidine was tested as follows. A crude protein extract of wild type *Neurospora* was tested for its ability to produce AIC-R-P from (a) PRPP + ATP and from (b) PRATP, both in the presence and absence of 0.5mM histidine. The results are summarised below.

Substrates ¹	AIC-R-P produced (E _{550mu})
PRPP + ATP	0.76
PRPP + ATP + his	0.02
PRATP	0.83
PRATP + his	0.88

¹ The concentrations of reactants were as described for the mixed extract assay method (see Chapter 3). PRATP was used at a concentration of 0.1umole/ml

Histidine at this concentration has no effect on the conversion of PRATP to AIC-R-P, but almost completely inhibits AIC-R-P production from PRPP + ATP. Thus the first reaction in the pathway is inhibited by histidine, but the following five reactions are not.

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